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REVIEW

Insights into myelodysplastic syndromes from current preclinical models

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Abstract

In recent years, there has been significant progress made in our understanding of the molecular genetics of myelodysplastic syndromes (MDS). Using massively parallel sequencing techniques, recurring mutations are identified in up to 80% of MDS cases, including many with a normal karyotype. The differential role of some of these mutations in the initiation and progression of MDS is starting to be elucidated. Engineering candidate genes in mice to model MDS has contributed to recent insights into this complex disease. In this review, we examine currently available mouse models, with detailed discussion of selected models. Finally, we highlight some advances made in our understanding of MDS biology, and conclude with discussions of questions that remain unanswered.

Key words: Myelodysplastic syndromes; Mouse models; Genetic mutations

Core tip: Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders. In recent years, we have witnessed a rapidly expanding

catalog of MDS candidate genes. Mirroring this, there has been an increased number of candidate genes employed to model MDS. Here, we aim to review currently available mouse models of MDS, highlighting models that are robust and well-characterized phenotypically with a particular focus on models that demonstrate close resemblance to the human disease.

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INTRODUCTION

Myelodysplastic syndromes (MDS) are neoplastic clonal disorders of ineffective hematopoiesis with an inherent risk of transformation to acute myeloid leukemia (AML)[1]. MDS typically manifests as increased intramedullary apoptosis of maturing clonal cells in a hyperproliferative and pro-inflammatory bone marrow[2-4]. Clinically, this is seen as peripheral blood cytopenia(s) with accompanying dysplasia in a hyper- or normocellular bone marrow.

It should be noted that apoptosis in the bone marrow is more prominent in low risk MDS, driven by an excess of pro-inflammatory cytokines and altered T cell response[5-7]. In advanced MDS, increased expression of BCL2 leads to resistance to apoptosis. Additionally, the acquisition of further molecular defects results in increased proliferation and blocked differentiation in myeloid progenitors, culminating in evolution to AML[8-11].

CLONAL HEMATOPOIESIS FROM A MDS STEM CELL

MDS is thought to arise from mutations in the hematopoietic stem cell/progenitor (HSPC) $CD34^+$ cell[12]. The founder mutation occurs in a MDS stem cell (or MDS initiating cell) that gives rise to clonal hematopoiesis. Support for this model of clonal architecture of MDS has been illustrated in several studies[13,14]. Delhommeau *et al*[13] isolated $CD34^+$ cells from MDS patients and identified *TET2* mutations only in a small fraction of immature $CD34^+CD38^-$ population with higher proportions detected in the $CD34^+CD38^+$ mature progenitors. The findings are in keeping with a model in which a *TET2* mutation arose in an immature progenitor cell and was passed on to its more mature progeny. In another study using whole genome sequencing, Walter *et al*[14] reported that about 85%-90% of unfractionated bone marrow cells were clonally derived from the MDS stage and persisted through to leukaemic transformation.

Of interest, whilst the MDS stem cell can establish clonal hematopoiesis, overt hematological manifestations of disease may be absent. It is likely that additional cooperating genetic and epigenetic events are required to drive disease progression and bring about a clinically apparent phenotype[15]. Indeed, age-related clonal hematopoiesis was first described in a group of healthy women over the age of 65. In approximately 23% of these women, a skewed pattern of X-chromosome inactivation was observed in cells taken from the peripheral blood, with some associated with *TET2* mutations[15,16]. More recently, whole exome sequencing identified the presence of clonal somatic mutations in genes that are recurrently mutated in hematological malignancy in the peripheral blood of ostensibly healthy elderly individuals[17-19]. Jaiswal *et al*[17] reported that the presence of somatic mutations was rare in individuals younger than the age of 40. However, the incidence of clonal mutations increases considerably with successive decades of life thereafter, with the frequency of mutations in individuals 60 years and older exceeding the incidence of hematological malignancy diagnosed in the general population. The most commonly mutated gene was *DNMT3A*, followed by *TET2, ASXL1, TP53, JAK2*, and *SF3B1.* These mutations persisted over time, and were associated with an increased risk, approximately 0.5%-1% per annum, of developing a hematological malignancy. In a second, independent cohort of subjects unselected with respect to hematological phenotypes, Genovese *et al*[18] found that more than 10% subjects aged 65 years or more had evidence of clonal hematopoiesis. In this population the most frequently mutated candidate driver genes were *DNMT3A*, *ASXL1*, *TET2*, *JAK2* and *PPMID* and the presence of a mutant clone was a risk factor for subsequent hematological malignancy or death. Finally, McKerrell *et al*[19] reported that the prevalence of clonal hematopoiesis doubled with each decade of life after the age of 50, rising from 1.5% in those aged 50-59 to nearly 20% in those 90 years and older. The most common mutations were *DNMT3A, JAK2, SRSF2* and *SF3B1.* Notably, spliceosome mutations at *SRSF2* P95, *SF3B1* K666 and K700 were exclusively observed in individuals older than 70 years. The striking degree of overlap between results of these studies with regards to the driver genes identified and the significantly heightened risk of hematological disease in individuals with clonal hematopoiesis serves to underline the generalizability of these findings.

CURRENT MOLECULAR INSIGHTS

MDS is a very heterogeneous disease, underscored by significant genomic instability and a complex genetic landscape. The catalog of MDS candidate genes is rapidly expanding with the application of modern techniques in detecting molecular lesions. However, the pathogenesis of MDS remains elusive. The hierarchical significance and functional interplay of the different mutations in the development and progression of the disease are areas of active investigation. Moreover, there is emerging evidence that MDS is not solely an intrinsic hematopoietic disease, with the niche, *i.e.*, bone marrow

microenvironment, also playing a role[5,6,20,21].

Recently, interrogation of MDS samples by massive parallel sequencing technology has allowed the identification of genetic mutations at single nucleotide resolution. Using this technique, mutations are apparent in up to 80% of cases, including many with a normal karyotype[22]. Recognition that recurrently mutated genes can be grouped according to the function of the proteins that they encode (epigenetic regulators, transcription factors, spliceosome components, *etc*.) has greatly improved our understanding of MDS pathogenesis[14,22,23].

CYTOGENETIC ABNORMALITIES

Cytogenetic analysis of MDS has been instrumental in the diagnosis and prognostication of MDS. Using conventional metaphase cytogenetics, abnormalities are found in up to 50% of patients with MDS[24], with a higher frequency of abnormal and complex cytogenetics (defined as the identification of three or more abnormalities in the karyotype) seen in therapy-related MDS.

As is the case for the recurrent mutations, many cytogenetic lesions seen in MDS are not exclusive to this disorder and also occur in other myeloid malignancies. However, copy number losses or gains are more frequent in MDS than balanced translocations, which tend to predominate in AML. The most common single cytogenetic aberrations include trisomy 8, del(5q), del(20q), and monosomy 7 or del(7q)[24-27]. It is thought that, in most cases, abnormalities detected by conventional cytogenetics are secondary genetic events resulting from genomic instability caused by earlier submicroscopic initiating or founder mutations[28]. Furthermore, cytogenetics only detects large-scale genomic changes, *i.e.*, loss or gain of a whole chromosome or most of a chromosome arm and as such, it is difficult to pinpoint candidate driver genes that are involved in a region of copy number change.

Although the abnormality rate for conventional cytogenetics is limited by the low-resolution inherent in using a microscope-based technology for the detection of genomic lesions, the information it provides remains clinically relevant. Conventional cytogenetics has a key role in identifying clonality. This can be central to MDS diagnosis which otherwise relies on subjective morphologic criteria. Importantly, possibly by virtue of the fact that cytogenetic abnormalities are seldom initiating events, cytogenetics provides powerful prognostic information in additional to diagnostic information, as demonstrated by the revised International Prognostic Scoring System (IPSS-R)[29].

MUTATIONS IN THE SPLICING MACHINERY

Pre-mRNA splicing is catalyzed by the spliceosome, a macromolecule composed of five small nuclear RNAs associated with numerous proteins to form small ribonucleoproteins. It is a highly dynamic structure, conferring accuracy in constitutively spliced exons and at the same time, allowing the flexibility for alternative splicing to generate genetic diversity and complexity.

Recently, whole genome and exome sequencing of human MDS samples has identified frequent somatic mutations in genes that encode components of the RNA splicing machinery, including *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*[22,30-32]. Indeed, RNA splicing is one the most common pathways targeted by mutations in MDS, with up to 50% of patients carrying a mutation in a gene encoding a spliceosome component[22].

Furthermore, mutations in the RNA splicing genes are mutually exclusive and are most often founding events. In fact, the mutant allele burden is typically between 40%-50%, indicating a dominant bone marrow clone that is heterozygous for the mutation[33,34]. Given the essential requirement for RNA splicing in generating protein diversity, biallelic mutations would be predicted to be lethal and evidence from mouse models largely supports this. Mutation hotspots in the three most frequently mutated genes, *SF3B1*, *SRSF2*, and *U2AF1*, have been identified. Almost all described mutations are missense, with no evidence of nonsense or frameshift changes, suggesting that they result in altered function rather than loss of function[30,32,35].

The mutations in individual spliceosome components are associated with different phenotypes and distinct clinical outcomes[22,34]. *SF3B1* mutations are found almost exclusively in patients with refractory anemia with ringed sideroblasts without or with thrombocytosis [refractory anemia with ringed sideroblasts (RARS) and RARS-T respectively], therefore proposing a causal link between mutation and ringed sideroblasts formation. Most patients with *SF3B1* mutations have good risk disease with a protracted clinical course and a low propensity to AML transformation[36]. On the other hand, *SRSF2* mutations are found mainly in patients with multilineage dysplasia and/or excess blasts and predict a high risk of leukemic evolution and poor survival[37-39]. *U2AF1* mutations have been reported in various MDS subtypes and found to predict high risk of progression to AML and hence, shorter survival[30,38].

The observation that spliceosome mutations are mainly founding mutations associated with different clinical outcomes led Papaemmanuil *et al*[22] to hypothesise that they give rise to initiating clones with different genetic predestination. Through specific cooperating genetic lesions, the initial driver mutations likely shape the trajectory of clonal evolution leading to more or less aggressive MDS phenotypes.

Spliceosome mutations are rarely found in childhood myeloid neoplasms. Moreover, they are rarely detected in the blood of young healthy individuals but increase in prevalence in an age-dependent manner in people aged 70 years and over, and confer an increased risk of myeloid malignancy[19]. These findings suggest RNA

splicing mutations are typically acquired with age and support the hypothesis that they occur early in disease pathogenesis[17-19].

MUTATIONS IN GENES INVOLVED IN EPIGENETIC REGULATION

Alterations in epigenetic processes, including DNA methylation, histone modifications and miRNA are now well-described and are pivotal in the pathogenesis of MDS.

Promoter-associated CpG island hypermethylation is seen in about 3%-5% of MDS, may occur early in the course of the disease and is associated with a more rapid progression to AML[40]. Recurrently mutated genes in MDS known to be involved in the regulation of DNA methylation include *TET2*, *DNMT3A* and *IDH1/2*[41-45].

Post-translational modification of histones plays an important part in epigenetic regulation. These proteins can be acetylated, methylated, and ubiquinated by a group of histone-modifying enzymes. Loss-of-function mutations occur in histone modifiers, such as *ASXL1* and *EZH2*, and they are associated with a poor prognosis and reduced survival[46-49].

MUTATIONS IN OTHER PATHWAYS

Mutations in signalling molecules, transcription factors, and *TP53* are often subclonal, driving disease progression and are associated with adverse clinical outcomes[22,50-54]. They tend to occur in advanced disease, with the exception of *TP53,* which may occur at an early stage in del(5q) MDS and therapy-related disease[55]. In the context of del(5q) MDS, mutated *TP53* is associated with lower response rates to lenalidomide treatment and an increased risk of leukaemic transformation[56].

MODELING MDS IN MICE

Animal models are valuable pre-clinical tools to advance our understanding of human diseases, as well as facilitating the development and evaluation of novel therapeutic agents. The laboratory mouse (Mus musculus) is the model of choice to phenocopy and to investigate the biology of human cancer for a variety of reasons including its small size, well-characterized physiology and rapid breeding cycle. Moreover, the frequently used C57BL/6J mouse strain has a fully sequenced genome, with 75% orthology to human[57].

In the case of MDS, mouse models are particularly useful to study the biology of this disease. By expressing MDS candidate genes in mice, the function of the various genes and their role in the pathogenesis and progression of the disease can be evaluated in detail. These models can also serve as a platform to identify and test novel therapeutic candidates. Additionally, they can also be used to evaluate the mechanism of action of therapies currently used in clinic, for example lenalidomide which is used in 5q- syndrome, and hypomethylating agents such as azacitadine and decitabine.

DIAGNOSING MDS IN HUMANS AND MICE

The diagnosis of MDS in humans is predominantly based on morphology. Based on the 2008 World Health Organisation (WHO) MDS classification, the minimum requirement for diagnosis include the presence of > 10% dysplasia morphologically, significant cytopenia in at least one lineage, and < 20% blasts[1]. The thresholds for significant cytopenias as recommended by the IPSS are hemoglobin < 10 g/dL, absolute neutrophil count < 1.8×10^9/L, and platelet count < 100×10^9/L. The only exception to meeting the minimum diagnostic prerequisites is evidence of clonality, *i.e.*, an abnormality from a pre-defined list of characteristic cytogenetic abnormalities is present. This is sufficient for a diagnosis of MDS, provided cytopenia is present and that AML has been excluded in the basis of the blast count[1].

Using the morphological criteria outlined above, MDS can be sub-classified depending on the number of lineages affected by cytopenias and dysplasia, and the enumeration of blasts in the bone marrow. Groups classified according to WHO criteria have prognostic significance, which can be refined further with cytogenetic information.

The presence of excess blasts, immediately places patients in a high-risk group. There are two groups, stratified according to blast count: Refractory anemia of excess blasts-1 (RAEB-1, 5%-9% marrow blasts) and RAEB-2 (10%-19% marrow blasts). The low-risk MDS comprises subtypes with only single-lineage cytopenia and dysplasia, and includes refractory anemia, and RARS. It should be noted that ringed sideroblasts can also be seen in other subtypes of MDS, and carry no independent prognostic significance. The intermediate-risk MDS, refractory anemia with multi-lineage dysplasia is associated with bi- or tri-lineage dysplasia.

Taking into consideration the diagnostic criteria and other salient characteristics described, the key features of human MDS that are desirable to recapitulate in mouse models include peripheral blood cytopenias, bone marrow dysplasia, ineffective hematopoiesis, a propensity to transform to secondary acute leukemia after a long latency, transplantable disease into secondary mice, and the ability to mimic therapeutic responses to treatments with established efficacy in human MDS.

It should be noted that mouse hematopoiesis differs from human hematopoiesis. In humans hematopoiesis is largely confined to the epiphyses from adulthood and compensatory hematopoiesis can occur in the bone marrow. In steady-state murine hematopoiesis, the bone marrow is 95% occupied leaving the spleen as the major site for compensatory blood cell production, and the spleen in mouse remains an important hematopoietic organ throughout life. As such, compensatory spleno-

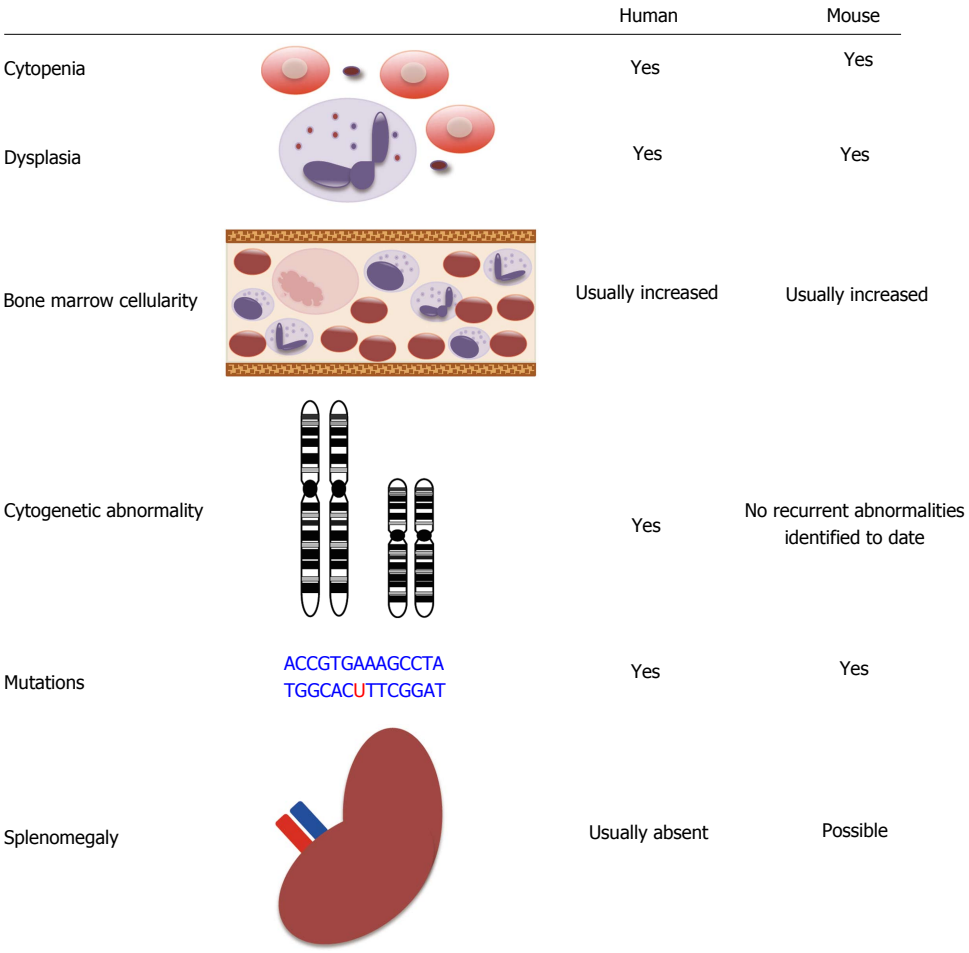

Figure 1 Key features of myelodysplastic syndromes in humans and mouse.

Table 1 Bethesda diagnostic criteria for myeloid dysplasia in mice

(1) At least one of the following
A: Neutropenia
B: Thrombocytopenia (without leucocytosis or erythrocytosis)
C: Anemia (without leucocytosis or thrombocytosis)
(2) Maturation defect in myeloid cells manifest as at least one of
A: Dysgranulopoiesis, dyserythropoiesis, or dysplastic megakaryocytes with or without increased myeloid immature forms or blasts
B: At least 20% myeloid immature forms or blasts
(3) Disorder is not AML
Subclassification
Myelodysplastic syndrome if meeting criteria (2)A
Cytopenia with increased blasts if meeting (2)B

Summarised from Kogan *et al*[60]. AML: Acute myeloid leukemia.

megaly in a cytopenic mouse may be considered the equivalent of bone marrow hypercellularity in a human and should not necessarily be regarded as evidence of a myeloproliferative neoplasm[58,59]. Thus, compensated or uncompensated anemia in a mouse with an MDS phenotype may be accompanied by splenomegaly. In addition, AML in mice will often involve both the bone marrow and the spleen, unlike in humans where leukemia tends to be confined to the bone marrow. Figure 1 illustrates the key features of myelodysplasia expected in both humans and mice, and also points out the differences between them.

DIAGNOSIS OF MDS IN MICE - THE BETHESDA CLASSIFICATION

Recognizing the need for consensus in the classification of murine hematopoietic lesions within the scientific community, the hematopathology subcommittee of the Mouse Models of Human Cancer Consortium (MMHCC) proposed diagnostic criteria for the classification of nonlymphoid hematopoietic neoplasms in mice (Table 1). There is a myeloid dysplasia category and within this, disease can be subclassified as either myelodysplastic syndrome or cytopenia with increased blasts[60]. To qualify as a myeloid dysplasia, acute nonlymphoid leukemia (*i.e.,* AML) must first be excluded. A diagnosis of AML should be applied if there are greater than 20% blasts, disseminated tissue infiltration and biologically aggressive disease that is rapidly fatal[60].

The defining criteria for myeloid dysplasia require the presence of cytopenia. Evidence of myeloproliferation in the form of erythrocytosis, leucocytosis and thrombocytosis must be absent. If there is morphologic evidence of dysplasia in at least one hematopoietic lineage, a myelodysplastic syndrome is said to be present

Table 2 Candidate genes used to model myelodysplastic syndromes in mice and their correlation to human myelodysplastic syndromes

Gene(s) studied	Chromosomal location	Frequency in human MDS	Equivalent human MDS subtype according to WHO 2008 classification	Ref.
Transcription factors				
NUP98-HOXD13	t(2;11)(q31;p15)	Rare	RCMD	[69]
RUNX1	21q22.3	15%-40%	RAEB	[105]
EVI1	3q26.2	Rare	RCMD	[136]
SALL4	20q13.2	15%-40%	RCMD	[139]
NPM1	5q35.1	About 4%	RCMD	[138]
Signalling molecules				
NRAS	1p13.2	About 20%	RAEB	[142]
BCL2	18q21.33	Unknown		
PTEN	10q23.3	Unknown	RCMD	[140]
SHIP	2q37.1	Unknown		
Epigenetic regulators				
TET2	4q24	20%-30%	RCMD/CMML	[115]
ASXL1	20q11.21	15%-20%	RCMD	[122,123]
EZH2	7q36.1	2%-6%	RCMD/MPN	[141]
MLL5	7q22.1	Unknown	No definitive MDS	[145]
RNA spliceosome				
SRSF2	17q25.2	15%-30%	RCMD	[98]
U2AF1	21q22.3	11%	No definitive MDS	[100]
SF3B1	2q33.1	10%-20%	RARS, RARS-T	[101]
Telomere function				
TERT	5p15.33	Unknown	RCMD	[124]
5q-				
RPS14	5q33.1	About 10%	5q-like	[91]
CD74-SMIM3 (NID67)	5q32-q33.1			[92]
SPARC	5q33.1			[97]
MIR145/146A	5q32-34			[143]
APC	5q22.2			[99]
CSNK1A1	5q32			[94]

WHO: World Health Organization; RARS: Refractory anemia with ringed sideroblasts; RARS-T: Refractory anemia with ringed sideroblasts and thrombocytosis; RCMD: Refractory anemia with multilineage dysplasia; RAEB: Refractory anemia with excess blasts; MPN: Myeloproliferative neoplasm; MDS: Myelodysplastic syndromes; CMML: Chronic myelomonocytic leukemia.

(Table 1). Morphological features of dysplasia are subtler in mice than in humans and can be difficult to identify. The MMHCC subcommittee lists features considered speculative evidence of dyspoiesis. In the erythroid lineage, dyserythropoiesis includes megaloblastic maturation, increased mitotic figures, multinucleation, and nuclear irregularity. Ringed sideroblasts are also a feature of dysplasia, but are rare in mice. Dysgranulopoiesis may manifest as hypogranular neutrophils, and lobated neutrophils as opposed to ring-shaped nuclei. For megakaryocytes, micromegakaryocytes, large megakaryocytes with unlobated nuclei or bizarre hypersegmented nuclei, and megakaryocytes with separated nuclei are all regarded as signs of dysplasia. Where AML has been excluded and morphologic dysplasia is lacking but there is cytopenia with more than 20% blasts in the bone marrow or spleen, the diagnosis cytopenia with increased blasts can be made. Provision is also made within the MMHCC criteria for disease in mice that very closely approximates a defined human MDS subtype. In this situation the disease may be labelled "myelodysplastic syndrome with features of a named human MDS"[60].

Little is known about the acquisition of cytogenetic abnormalities in mouse models of MDS. Thus, recurrent cytogenetic abnormalities cannot be used to aid the diagnosis and classification of disease in mice, in the way that they can be used in humans.

AVAILABLE MOUSE MODELS OF MDS

There are more than 20 published mouse models of MDS in the literature, and they are summarised in Tables 2 and 3. Several strategies have been employed to create these models. They include genetic engineering of mouse hematopoietic cells using knock-in or knock-out strategies, and xenotransplantation of human MDS cells, the latter of which has proven to be technically difficult.

Mirroring the rapidly expanding catalog of MDS candidate genes, there have been an increasing number of genetically modified mice being reported as MDS mouse models (Figure 2). These models mostly examine the effects of a single gene modification in the pathogenesis of MDS, and many of them displayed features reminiscent of MDS. Considering the genetic complexity inherent to MDS, it is not surprising that a single unifying model that faithfully mimics the MDS phenotype in its entirety is still lacking. More recently, there have been efforts to incorporate more than one mutation in modeling MDS, which has certainly provided insights into the functional interactions of different genes in the

Table 3 Published mouse models of myelodysplastic syndromes

Gene(s) studied	Model/technique	Tractability to human MDS								Ref.
		Anemia	Multi-lineage cytopenias	Dysplasia	Bone marrow cellularity	HSPC skewing	Secondary leukemia	Latency to leukemia	Survival	
NUP98-HOXD13	Transgenic	Yes	Yes	Yes	Hyper-	Yes	Yes[2]	14 mo	-	[69]
Cd74-Nid67	Large scale chromosomal deletion, RPS14 haploinsufficient	Yes	No	Yes	Hypo-	Yes	No	-	[1]	[92]
SPARC	Knock-out	No	No	Yes	[1]	[1]	No	-	[1]	[97]
MIR-145, MIR146a	Retroviral transduction	No	No	Yes	[1]	[1]	Yes	4-14 mo	-	[143]
APC	Transgenic, haploinsufficient	Yes	No	Yes	[1]	Yes	No	-	3-8 mo	[99]
Csnk1a1	Transgenic, inducible	Yes	Yes	Yes	Yes	Yes	No	-	[1]	[94]
Srsf2	Transgenic, inducible	Yes	Yes	Yes	Normal	Yes	No	-	[1]	[98]
U2af1	Transgenic, inducible	No	No	No	Normal	Yes	No	-	-	[100]
Sf3b1	Transgenic, haploinsufficient	Yes	No	Yes	[1]	[1]	No	-	About 12 mo	[101]
RUNX1-D171N	Retroviral transduction	Yes	Yes	Yes	Normal to hyper-	[1]	Yes	4-13 mo	-	[105]
RUNX1-S291fsX300	Retroviral transduction	Yes	Yes	Yes	Normal to hyper-	[1]	Yes	4-13 mo	-	[105]
RUNX1S291fs/Ezh2	Retroviral transduction	Yes	Yes	Yes	Variable	[1]	No	-	262 d	[125]
Tet2	Transgenic, hypomorphic	Yes	Yes	Yes	Hyper-	Yes	No	-	11 mo	[115]
Tet2/Ezh2	Transgenic, inducible	Yes	Yes	Yes	Hyper-	Yes	No	-	10 mo	[141]
ASXL1	Transgenic, inducible	Yes	Yes	Yes	Hypo-	Yes	No	-	Median 50 wk	[122]
Asxl1	Transgenic, constitutive	Yes	Yes	Yes	Normal to hyper-	Yes[3]	16 mo	8-42 d	-	[123]
TERT	Transgenic, inducible	Yes	Yes	Yes	Hyper	Yes	Yes	[1]	12 mo	[124]
Evi1	Retroviral transduction	Yes	Yes	Yes	Hyper-	[1]	No	-	10-12 mo	[136]
SALL4	Transgenic	Yes	Yes	Yes	Hyper-	Yes	Yes	6 wk to 12 mo	-	[139]
Npm1	Transgenic, haploinsufficient	No	No	Yes	Hyper	No	Yes	24 mo	-	[138]
NRASD12-BCL2	Transgenic, inducible/ constitutive	[1]	No	Yes	[1]	Yes	Yes	3-6 mo	-	[142]
Pten/Ship	Transgenic, Pten +/- Ship -/-	Yes	Yes	[1]	Hypo	Yes	No	-	5 wk	[140]
Dido	Knock-out	Yes	No	Yes	[1]	[1]	No	-	1	[137]
Arid4a	Knock-out	Yes	Yes	Yes	Hyper-	[1]	Yes	5 mo	-	[144]
Mll5	Knock-out	Yes	No	No	[1]	[1]	No	-	-	[145]

[1]Denotes not reported, denotes not applicable; [2]Some mice developed pre-T cell acute lymphoblastic leukemia; [3]Only 1 of 18 mice developed AML. HSPC: Hematopoietic stem and progenitor cells; MDS: Myelodysplastic syndromes; AML: Acute myeloid leukemia.

biology of MDS.

Amongst the published mouse models, anaemia was the most common cytopenia reported with a significant proportion showing multilineage cytopenia including pancytopenia in some models. Accompanying dysplastic changes were often noted in more than lineage. It should be noted that unlike the number of cytopenias which is indicative of MDS severity, the extent of dysplasia carries no prognostic implications. Several of these models also showed alterations in the hematopoietic stem cell (HSC) and a propensity to transform to secondary AML, further improving their tractability to the human disease.

XENOGRAFT MOUSE MODELS OF MDS

The establishment of immunodeficient mice harbouring malignant human xenografts is an attractive approach to model and study malignancy of the hematopoietic system. Establishing human malignant cells in the mouse host has been technically challenging, but has proven feasible in hematological disorders such as AML and acute lymphoblastic leukemia (ALL). In contrast, the propagation of MDS clones has been met with limited success. This has been attributed to a number of factors including host anti-tumor/human immune response, inadequate microenvironment for tumor growth or survival, and toxicity from *ex vivo* manipulation of malignant cells.

Techniques have improved considerably over the last ten years, and the generation of transgenic severe combined immunodeficient (SCID) mice expressing human granulocyte-macrophage colony-stimulating

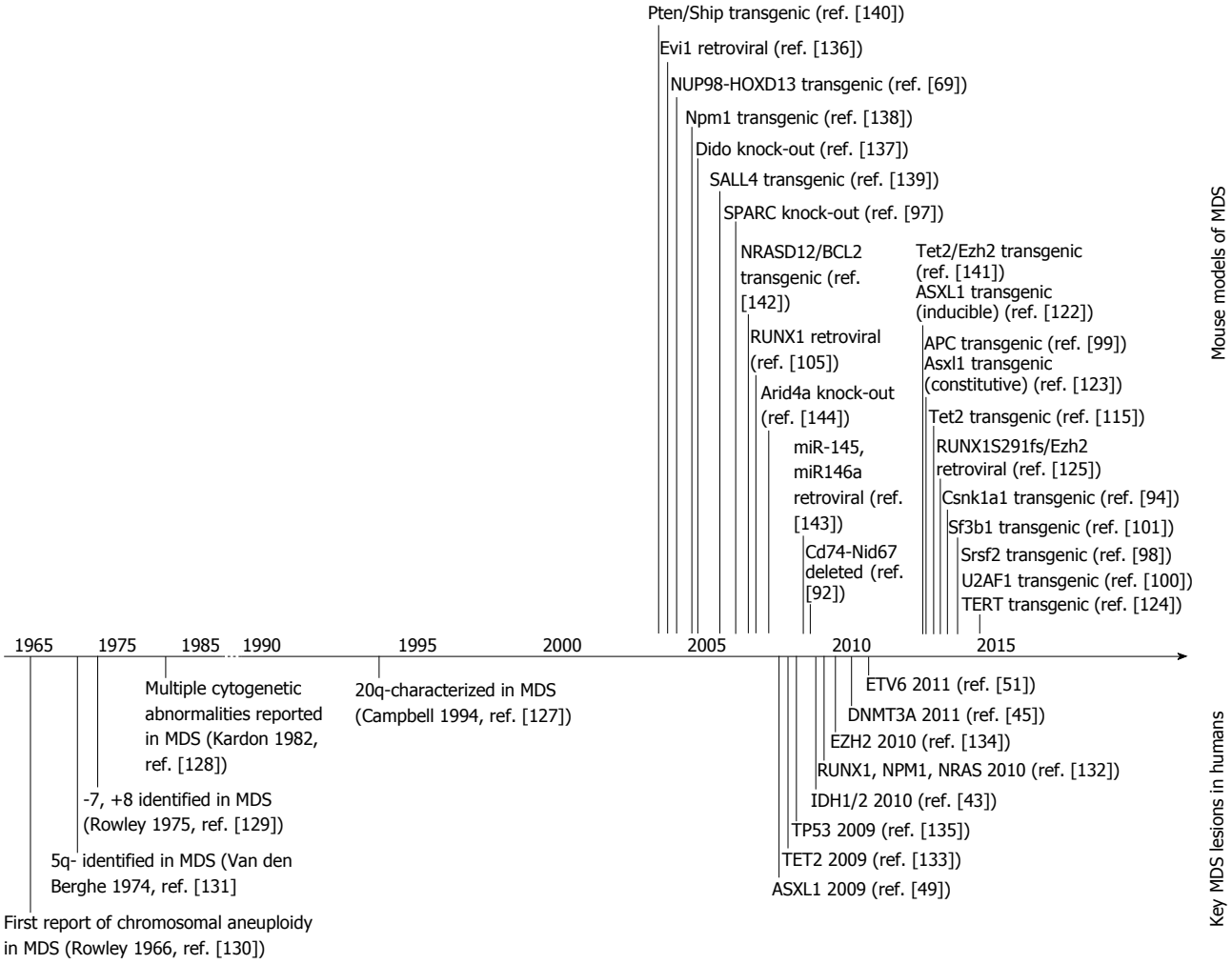

Figure 2 Time-line showing key genetic aberrations identified in myelodysplastic syndromes and published mouse models. MDS: Myelodysplastic syndromes.

factor and interleukin-3 (IL-3) has improved the engraftment of an immortalized cell line derived from a patient with MDS (F-36P). Engraftment was further improved with the pre-administration of IL-2 receptor antibodies, which suppressed natural killer (NK) cell function[61].

This led to the development of non-obese diabetic (NOD)-SCID mice that have reduced natural killer cell function, as well as deficiencies in T- and B-cells. However, injection of progenitor cells from MDS patients with del(5q) and trisomy 8 into these mice showed poor engraftment[62,63]. Although one of seven mice with del(5q) showed low-level of engraftment, no clinical phenotype of MDS was observed. In another study, bone marrow cells from MDS patients and healthy controls were injected into sublethally irradiated NOD-SCID mice, with or without human cytokines[64]. Cells from patients with MDS demonstrated reduced marrow repopulating ability compared to healthy controls. Moreover, previously observed karyotypic abnormalities could not be identified in recipient mice, suggesting that most of the engrafted human cells were derived from normal bone marrow. Taken together, these studies showed that the NOD-SCID environment could not reliably and reproducibly support the expansion of human MDS cells.

The generation of NOD-SCID beta2-microglobulin-null mice (NOD-SCID-B2m$^{-/-}$) that have suppressed NK cell function, but express human cytokines and steel factor (c-kit ligand) has allowed the repopulation of MDS clones[65]. However, the level of engraftment was less than 1% of nucleated cells, and the mice did not develop clinical disease.

More recently, intravenous co-administration of the human marrow stromal cell line HS27a with CD34$^+$ MDS cells in NOD-SCID gamma (NSG) mice was explored and showed considerable promise with engraftment documented in 44 of 46 (95%) mice[66]. Co-localisation of the stroma and CD34$^+$ cells were seen in the spleen of the recipient mice, and furthermore, these cells also engrafted successfully in secondary NSG recipients. This study suggested that HS27a stromal cells in direct contact with the hematopoietic precursors supported their propagation. In another study, overproduction of niche factors such as CDH2 (N-Cadherin), IGFBP2, VEGFA, and LIF enhanced the expansion of MDS mesenchymal stromal cells, highlighting the complexity of the

disease and that it requires the engagement of both the hematopoietic and stromal elements to propagate[67].

Collectively, these studies demonstrate ongoing progress in the development of xenograft models of MDS. Overall, more robust and more consistent engraftment of MDS cells that can result in clinical disease is needed to improve the utility of this approach. While poor engraftment of MDS cells remains the main drawback, the requirement of an immunocompromised host with this technique makes it unsuitable for the bone marrow niche to be examined.

GENETICALLY ENGINEERED MOUSE MODELS OF MDS

Generally, genetic engineering of hematopoietic cells of mice has been accomplished using two approaches. The first approach involves *in vitro* transduction of bone marrow with viral overexpression/shRNA vectors and subsequent transplantation into a histocompatible, irradiated host. The second approach involves modification of the mouse germline to generate mice with altered expression of a particular gene of interest. These approaches can be further refined with the employment of the Cre-Lox recombination system which allows gene expression to be controlled in a temporal, cell type and spatial manner[68].

Indeed, conditional knock-in mice are currently the most favored technique in generating mouse models of MDS. The gene in question can be manipulated easily and importantly, is expressed at more physiological levels. The host is often immunocompetent, and the bone marrow niche can also be examined. In comparison, retroviral models require transplanting transduced cells into lethally irradiated recipients and hence, results in supraphysiological levels of gene expression. Moreover, the bone marrow niche is altered through the process of irradiation and transplantation. As a result, the observed effects of altered gene expression in this context is not entirely representative.

In the subsequent sections, we will focus our discussions on selected mouse models of MDS (Table 3). We will highlight models that are robust and well-characterized phenotypically, as well as models that illustrated different genetic lesions that are clinically relevant. We have particularly focused on the mouse models that demonstrate synergy to human disease.

MODELLING CYTOGENETIC ABNORMALITIES - CHROMOSOMAL TRANSLOCATIONS

NUP98-HOXD13

Of all the approaches that have been explored to model MDS to date, the *NUP98-HOXD13* mouse model is the best established, and perhaps the only published model that has been able to recapitulate many of the key features of MDS[69].

The *NUP98-HOXD13* involves the fusion of two genes: Nucleoporin protein, *NUP98,* with homeobox D13, *HOXD13*. The *NUP98-HOXD13* fusion gene, which is generated by the chromosomal translocation t(2;11)(q31;p15), was initially identified in a patient with therapy-related MDS (t-MDS)[70]. Although numerous partner genes of *NUP98* have been reported in various hematopoietic malignancies, balanced translocations are rare in MDS[71,72], and there are very few cases of MDS bearing the t(2;11) reported in the literature[73].

The first reported *NUP98-HOXD13* mouse model was established by a retroviral system. Pineault *et al*[74] constructed *NUP98-HOXD13* (*ND13*) cDNA using *ND13* cDNA fragment isolated from a patient with t-MDS, and transplanted transduced murine bone marrow cells into irradiated recipient mice. *ND13* expressing mice showed a preferential increase in myelopoiesis at the expense of B and T-cell lymphopoiesis, and developed overt features of myeloproliferative disease five months post-transplant. The mice did not progress to AML, however accelerated leukemic transformation was observed when the *HOX* cofactor, *Meis1,* was co-transduced with *ND13*.

Subsequently, conditional *NUP98-HOXD13* (*NHD13*) transgenic mice were developed, using a vav promoter to drive *NHD13* expression in hematopoietic tissues[69]. At 4 to 7 mo, these mice developed anemia and neutropenia, with variable degree of macrocytosis and thrombocytopenia. This was accompanied by normal or hypercellular bone marrow with dysplasia observed in multiple lineages. In line with human MDS, about half of *NHD13* mice with MDS developed acute leukemia, typically at 10 to 14 mo of age. Although AML was the most common type of leukemia reported, several mice also developed precursor T-cell lymphoblastic lymphoma/leukemia (T-ALL), which is rarely reported in human MDS. The T-ALL predisposition may be related to increased levels of *Hoxa* cluster genes, such as *Hoxa7* and *Hoxa9,* which have an association with T-ALL[75].

In addition to the key features of MDS described above, it is noteworthy that the *NHD13* mice showed marked reductions in undifferentiated lineage negative (lin^{neg}) hematopoietic precursors *in vitro* and *in vivo,* which are comparable to results from studies performed on MDS patients[76-79]. This was further accompanied by impaired differentiation with the majority of the *NHD13* lin^{neg} cells undergoing apoptosis, which is a salient feature in human MDS. Gene expression profiling of $Lineage^-$, $c\text{-}kit^+$, $Sca\text{-}1^-$ (LKS^-) myeloid progenitor cells from 3-mo-old NHD13 mice that displayed macrocytic anemia showed 3.6-fold reduction in BCL2[79]. Enforced expression of BCL2 inhibited apoptosis at the HSPC level, rescued the macrocytic anemia and interestingly, also abrogated leukaemic transformation[79].

The *NHD13* model has also been used to identify secondary mutations that lead to acute leukemia in the mouse. An increased frequency of *Nras* and *Kras* mutations has been noted in *NHD13* mice that pro-

gressed to leukemia[80]. In contrast, *Npm1, Trp53, Runx1, Kit,* and *Flt3* mutations were not increased, and *Meis1,* which induces leukaemic transformation in the retroviral model, was not altered in the transgenic model[69,80].

The initial transgenic *NHD13* study was performed using FVB/N background mice. The entire study was subsequently repeated with C57Bl/6 mice with similar findings, demonstrating that effects of the transgene were reproducible and not compounded by the genetic background of the mice[69]. In comparison, there are considerable differences between the transgenic and retroviral transduction models, which may be explained by *ex vivo* manipulation of cells, differences in mouse strain, amount of overexpression of NHD13 achieved and/or the differential effects of *ND13* retrovirus on the bone marrow hematopoietic stem/progenitor cells and their subsequent bone marrow reconstitution.

MODELLING CYTOGENETIC ABNORMALITIES - CONTIGUOUS GENE DELETION SYNDROMES

5q-

Deletion of the long arm of chromosome 5, del(5q), is the most common cytogenetic abnormality found in MDS, accounting for approximately 10%-15% of cases[24,81]. The 5q- syndrome is recognized as a distinct clinical entity in the 2008 WHO classification, and is defined by del(5q) being the sole karyotypic abnormality[1]. It has a female preponderance, and a distinct phenotype characterized by refractory anemia with normal or increased platelet count, erythroid hypoplasia, hypolobated megakaryocytes, < 5% blasts, and lenalidomide responsiveness. MDS associated with isolated del(5q) carries a good prognosis with a low risk of transformation to AML[82-84].

There are two distinct commonly deleted regions (CDR) in 5q- syndrome. The more distal CDR is mapped to a 1.5-megabase region between bands 5q31 and 5q33[85,86].

The CDR contains 24 known genes, 16 predicted genes, and four known microRNAs (*MIR584*, *MIR143*, *MIR145* and *MIR378A*)[85]. The more proximal CDR contains 18 genes, and has been associated with more advanced MDS and AML[87,88]. Point mutations in *CSNK1A1* occur in approximately 5% cases of 5q- syndrome[89], however point mutations have not been identified in the remaining coding genes in the distal CDR. The absence of point mutations in the majority of cases suggests that haploinsufficiency of one or more genes, or the epigenetic inactivation of a retained tumor suppressor allele are responsible for the disease phenotype[90]. The study of the haploinsufficient effect of the coding genes in the distal CDR led to the identification of *RPS14*, which encodes a component of the 40S ribosomal subunit. Reduced *RPS14* expression leads to defects in ribosome biogenesis and protein translation, resulting in apoptosis of erythroid cells and macrocytic anemia[91]. Furthermore, this phenotype was rescued *in vitro* by enforced expression of *RPS14* in $CD34^+$ bone marrow cells derived from 5q- syndrome patients indicating that haploinsufficiency of RPS14 is responsible for the erythroid phenotype in the 5q- syndromes.

Subsequently, Barlow *et al*[92] generated a mouse model using Cre-loxP recombination to delete a large region on chromosome 18 flanked by the *Cd74* gene and small integral membrane protein 3 (Smim3, also known as *Nid67)* in the mouse. The haploinsufficient region in this model is syntenic to a region within the 5q- CDR in humans that contains *RPS14*. The *Lmo2Cre*$^+$ *Cd74-Nid67* deleted mice developed severe macrocytic anemia, prominent dyserythropoiesis and monolobated megakaryocytes, in keeping with the characteristics of 5q- syndrome. On the other hand, these mice developed thrombocytopenia, which is generally not seen in 5q- syndrome unless in the context of disease progression or leukaemic transformation.

In addition, the *Cd74-Nid67* deleted mice had hypocellular bone marrow with 50%-60% reductions in cell numbers, accompanied by defective production of progenitor cells with proportionally reduced trilineage colony-forming potential *in vitro* compared to controls. Deletion of various segments of mouse chromosomes 11 and 18 syntenic to other regions of the 5q- CDR and exclusive of *Rps14* did not give rise to red cell phenotype. The deleted region of this *Cd74-Nid67* mouse contains five candidate genes (*Synpo, Myoz3, Rbm22, Dctn4* and *Nid67*) in addition to *Rps14*. Nonetheless, the fact that it was only mice in which the region containing *Rps14* was deleted that had a macrocytic anaemia phenotype was consistent with the findings of Ebert *et al*[91] that *RPS14* is the key contributor to the erythroid phenotype seen in 5q- syndrome.

Of interest, *Cd74-Nid67* deleted mice showed increased intracellular Trp53 (p53) in their immature progenitor cells. Although not statistically significant, there was a trend to an increase in annexin-V^+ (early apoptotic) cells in the *Cd74-Nid67* deleted marrow compared to control mice, suggesting that cell cycle arrest and apoptosis was probably enhanced by the stabilization of Trp53 in these cells. Following this, Barlow *et al*[92] elegantly showed that homozygous *Trp53* deletion rescued the progenitor deficits and normalized the peripheral blood phenotype observed in *CD74-Nid67* deleted mice. Taken together, these findings suggest that the loss of *RPS14* results in impaired ribosomal biogenesis and consequently TP53 activation, leading to increased apoptosis and erythroid hypoplasia.

More recently, there have been two studies that explored the role of casein kinase 1A1 (*CSNK1A1*) in the pathophysiology and treatment of 5q- syndrome[93,94]. *CSNK1A1* is located in the distal CDR, and encodes a serine/threonine kinase. Gene expression analysis of $CD34^+$ cells from MDS patients with del(5q) demonstrated the haploinsufficiency of *CSNK1A1* with approximately 50% of normal expression[90]. Furthermore, studies in

solid organ malignancies showed that *CSNK1A1* acts as a tumour suppressor gene through regulation of the β-catenin pathway, and also regulates TP53 activity[95,96].

Schneider *et al*[94] generated an *Mx1Cre*-inducible *Csnk1a1* exon 3 knock-out mouse model, and demonstrated that activation of β-catenin activity was proportional to the allelic loss of *Csnk1a1*. Accumulation of β-catenin was noted in both hematopoietic and stromal cells consistent with the expression of *Mx1Cre* in bone marrow stroma[97], with more pronounced expression in homozygous knock-out mice. As such, the function of mesenchymal stem cells in supporting hematopoiesis is significantly impaired in the knock-out mice, with inactivation of β-catenin rescuing the effect.

Homozygous knock-out mice (*Csnk1a1*$^{-/-}$ *Mx1Cre*$^{+}$) rapidly developed profound pancytopenia, fulminant bone marrow failure, multi-organ ischemia and death in 5-17 d, demonstrating the critical role of *Csnk1a1* in hematopoiesis[94]. Moreover, there was accumulation of Trp53 and its target, Cdkn1a (p21), leading to induction of early and late apoptosis with a marked decrease in cells in G0 and a significant increase in cells in S/G2/M phases in keeping with stem cell exhaustion.

Transplantation of bone marrow cells from heterozygous knock-out mice (*Csnk1a1*$^{-/+}$) into lethally irradiated mice showed that transplant recipients developed normal to hypercellular bone marrow, accompanied by increased and mildly dysplastic hypolobated megakaryocytes, as well as thrombocytosis over time[94]. Additionally, non-competitive transplantation of *Csnk1a1*$^{-/+}$ bone marrow showed increased proportions of HSC-enriched Lineage^{-} ckit^{+}Sca-1^{+} (LKS^{+}) cells in contrast to reduced Lineage^{-} ckit^{+}Sca-1^{-} (LKS^{-}) myeloid progenitor cell populations. This was further demonstrated to be related to exit of *Csnk1a1*$^{-/+}$ HSCs from quiescence, with reduced cells in G0 and a significantly increased proportion of cells in the cycling G1 fraction and S phase, which was due to increases in β-catenin activity and expression of cyclin D1. A competitive advantage was demonstrated in *Csnk1a1* haploinsufficient bone marrow using long-term repopulating assays, where haploinsufficient cells were significantly more abundant than controls at 16 wk following primary and secondary transplant, specifically with increased LKS^{+} cells and increased myeloid progenitor cells and CD3^{+} T cells. Collectively, the hypolobated megakaryocytes and self-renewal cells in *Csnk1a1*$^{-/+}$ cells highlight the role of *Csnk1a1* haploinsufficiency and β-catenin in the megakaryocyte phenotype and clonal expansion that occur in 5q-syndrome.

Correlating this clinically, the group performed whole-exome sequencing on MDS samples, and identified a small proportion of patients (3 of 43) with somatic mutations in *CSNK1A1.* All three patients had mutations that resulted in the same amino acid change (E98K or E98V), and all had wildtype *TP53*. Analysis by SNP array showed a high variant allelic frequency of the del(5q) MDS clone, indicating that the deletion of chromosome 5q preceded *CSNK1A1* mutation in remaining allele.

The functional consequence of *CSNK1A1 E98V* mutation was then examined by retroviral transduction of mutant cDNA in *Csnk1a1*$^{-/-}$ *Mx1Cre*$^{+}$ haematopoietic cells and transplantation into lethally irradiated recipients. Firstly, it was noted that cDNA overexpression of *CSNK1A1 E98V* mutation rescued the HSC ablation in *Csnk1a1*$^{-/-}$ *Mx1Cre*$^{+}$ cells. Secondly, in comparison to cells transduced with the WT cDNA, *CSNK1A1 E98V* transduced cells have increased nuclear β-catenin activity but do not cause increased p53 activation. Collectively, these findings provided evidence that that *CSNK1A1 E98V* mutation do not cause of loss of function, but conversely confer selective advantage and drives clonal dominance of del(5q) MDS cells.

Finally, the group showed that *Csnk1a1* haploinsufficiency sensitizes cells to casein kinase 1 inhibition with D4476. Using purified myeloid progenitors, *Csnk1a1* haploinsufficient cells demonstrated reduced viability and increased apoptosis compared to control cells at a range of D4476 drug concentrations.

In another study by the same group, Krönke *et al*[93] showed that reduced *CSNK1A1* levels sensitize hematopoietic cells to lenalidomide. Inhibition of cell growth and proliferation was observed in the presence of lenalidomide using transduced primary human CD34^{+} hematopoietic stem and progenitor cells with shRNA knockdown of *CSNK1A1*. Overexpression of *CSNK1A1* in bone marrow samples of MDS patients with del(5q) led to reduce *in vitro* sensitivity to lenalidomide in 3 of 5 patients, which correlated with the clinically observed cytogenetic response. In contrast, overexpression of *CSNK1A1* had no effect in normal donors and in MDS with a normal karyotype, highlighting the therapeutic window for selectively targeting MDS cells by lenalidomide in del(5q) MDS.

Lenalidomide induces the ubiquitination of casein kinase 1A1 (CK1a) *via* the E3 ubiquitin ligase CUL4-RBX1-DDB1-CRBN (known as CRL4^{CRBN}) in a species-specific manner. Mice are insensitive to the teratogenic effects of thalidomide. Similarly, *Csnk1a1*$^{-/+}$ murine cells are insensitive to lenalidomide because degradation of CK1a does not occur after binding of lenalidomide to mouse Crbn. This is due to a single amino-acid difference between cereblon in mice and humans. Substitution of isoleucine for the human valine at position 391 of mouse Crbn (CrbnI391V) is sufficient to rescue lenalidomide sensitivity. This data illustrates the importance of taking into account and leveraging differences between mice and humans when using mice to model human diseases.

MODELLING SINGLE GENE MUTATIONS AND OTHER SUBMICROSCOPIC CHANGES

Modelling mutations in the RNA splicing genes

Somatic mutations in components of the 3' pre-mRNA splicing machinery are common, and are frequently early pathogenetic events in MDS. However, the functional

contribution of these mutations in the evolution of MDS remains to be delineated. It is unclear whether mutation in a splicing factor affects the splicing of a single gene or large number of genes, or even whether the downstream impact of these mutations is mRNA splicing-dependent.

SRSF2

SRSF2 is mutated in 20%-30% cases of MDS, and about 50% cases of chronic myelomonocytic leukemia (CMML). Importantly, it is associated with an inferior prognosis[32,35]. SRSF2 is a member of the serine/arginine-rich protein family, and binds to exonic splicing enhancer sequences (ESEs) within pre-mRNA through the RNA recognition motif domain.

To study the functional impact of *SRSF2* mutations on hematopoiesis or splicing, Kim *et al*[98] generated a hematopoietic-specific conditional *Srsf2* knock-in mouse model with the commonly occurring SRSF2^{P95H} mutation. Heterozygous transgenic mice were generated and crossed to *Mx1-cre* mice. Bone marrow mononuclear cells from *Srsf2* wildtype (WT), *Srsf2*fl/WT (heterozygous deletion of one copy of *Srsf2*), *Srsf2*fl/fl (homozygous floxed mice for both copies of *SRSF2*), and *Srsf2*P95H/WT were transplanted into lethally irradiated recipients, followed by polyinosine-polycytosine treatment four weeks later.

Mice transplanted with BM cells harbouring the homozygous *Srsf2* deletion or the *Srsf2*P95H/+ mutation developed significant anemia and leucopenia at 18 wk post-transplant[98]. In addition to the observed bicytopenia, *Srsf2*P95H/+ mice also displayed macrocytic erythrocytes, accompanied by normocellular bone marrow with multilineage dysplasia in the erythroid and myeloid lineages, mimicking features of human MDS. In contrast, and consistent with the original published full knock-out model, homozygous *Srsf2* deletion led to profound bone marrow aplasia without evidence of dysplasia morphologically.

Moreover, *Srsf2*P95H/+ mice showed increased LKS$^+$ cells, increased early apoptosis and increased proportion of cells in the S-phase of the cell cycle[98]. The increase in HSC and progenitor cells in conjunction with peripheral cytopenia is suggestive of impaired differentiation. Flow cytometry showed that the observed peripheral leucopenia was predominantly due to reduced B-lymphopoiesis. *Srsf2*P95H mice also had reduced early erythroid progenitors with reduced pre-MegE and pre-colony-forming units, erythroid. Of note, none of the non-transplanted *Srsf2* P95H mice developed overt MDS phenotypes or acute leukemia even well past a year of monitoring and the primary phenotypes reported were all present only in the context of transplant studies.

Subsequently, the authors showed that *Srsf2* P95H mutation altered the normal function of SRSF2, instead of resulting in haploinsufficiency or a dominant negative form. Using RNA sequencing, they found that *Srsf2* mutation led to genome-wide alteration, rather than loss, of its normal ESE recognition activity. Wild-type SRSF2 recognizes the consensus binding sequences CCNG and GGNG with similar affinity[99]. In contrast, *SRSF2* mutation resulted in preferential recognition of cassette exons containing C- *vs* G-rich ESEs. This was further supported by biochemical analysis which demonstrated that SRSF2 mutation was associated with a conformational change in its RNA recognition motif domain, consequently altering the interaction specificity between SRSF2 and pre-mRNA.

At a functional level, the authors proposed that this drives recurrent missplicing of key hematopoietic regulators, including SRSF2 mutation-dependent splicing of *Ezh2*. Missplicing of *Ezh2* leads to nonsense-mediated decay, reduced Ezh2 protein expression and in turn, contributes to impaired hematopoietic differentiation. *SRSF2* and *EZH2* mutations are known to be mutually exclusive in MDS, and findings in this study have provided a potential mechanistic explanation for this observation[22]. Finally, the authors showed that overexpression of normally spliced *Ezh2* cDNA in progenitor cells from *Srsf2* P95H/+ mice partially rescues the hematopoietic defect induced by *SRSF2* mutation in methylcellulose colony forming cell assays.

U2 small nuclear RNA auxiliary factor 1

U2 small nuclear RNA auxiliary factor 1 (*U2AF1*) is one of the most commonly mutated genes in MDS, and can be found in approximately 11% of patients[30,32]. It is typically a founder mutation, and is associated with a less favorable prognosis with a high risk of transformation to AML[14,22]. Previous studies using a retroviral overexpression model of mutant *U2AF1* have demonstrated that transduced murine bone marrow cells have reduced repopulating potential *in vivo*[32].

More recently, Shirai *et al*[100] generated a doxycycline-inducible transgenic mouse model with the most commonly identified *U2AF1* (S34F) mutation. Human cDNA encoding for *U2AF1* (S34F) or *U2AF1* (WT) were inserted into the *Col1a1* locus of KH2 mouse embryonic stem cells, which contain the M2rtTA tetracycline-responsive transactivator protein (rtTA) ubiquitously expressed from the *Rosa26* locus to allow for induction of the transgene. Bone marrow cells from transgenic mice were transplanted into lethally-irradiated wild-type congenic mice, and allowed to engraft prior to the induction of transgene expression with doxycycline treatment for 12 mo.

Peripheral blood leucopenia was observed in the *U2AF1* (S34F) mice after one month of doxycycline treatment, and appears to be related to B-lymphopenia and monocytopenia based on flow cytometry. Leucopenia persisted up to 12 mo, with white cell counts recovering to levels similar to that of controls following withdrawal of doxycycline treatment, suggesting a relationship between expression of mutant *U2AF1* and the phenotype seen.

Strikingly, *U2AF1* (S34F) mice showed increased proportions of HSPC in the bone marrow, particularly in the multipotent progenitors and common myeloid

progenitor (CMP) compartments. Moreover, there was increased cell cycling in the LKS^+ population as evidenced by increased Ki67 staining. Overall, the bone marrow cellularity of *U2AF1* (S34F) mice was not significantly different to controls. In mature cell lineage analysis, B-lymphopenia and monocytopenia were also noted in bone marrow. This appeared to be due to neutrophilia and increased in apoptosis of the monocytes.

Interestingly, there was no morphological evidence of dysplasia despite 12 mo of doxycycline treatment. Whilst there were convincing features of perturbed hematopoiesis, and bone marrow characteristics reminiscent of MDS in *U2AF1* (S34F) mice, they do not meet the Bethesda criteria for a myelodysplastic syndrome. They also failed to develop AML. It would be interesting to establish whether other cooperating mutations such as *ASXL1* give rise to MDS and AML in these mice. However, this work remains important as it has shed light on the effects of *U2AF1* mutation on hematopoiesis. In addition, the authors used RNA sequencing data to identify a splice junction sequence-specific pattern of altered splicing induced by *U2AF1* mutation. Exons skipped more frequently and alternative splice sites used more often than canonical splice sites by *U2AF1* (S34F) were enriched for a uracil in the minus 3 position relative to the AG dinucleotide, consistent with published reports of mutant *U2AF1*-associated splicing abnormalities seen in other malignancies[101]. Moreover, integration with human RNA sequencing datasets determined that common mutant *U2AF1*-induced splicing alterations are enriched in RNA processing genes, ribosomal genes, and recurrently mutated MDS and acute myeloid leukemia-associated genes. Taken together, this supports the hypothesis that *U2AF1* mutation alters downstream gene isoform expression, thereby contributing to abnormal hematopoiesis in MDS.

Collectively, studies modelling RNA splicing mutations in mice have provided insights into genetic lesions affecting spliceosome function and mRNA splicing. These findings have already improved our mechanistic understanding of the role of spliceosome mutations in altering the transcriptome, and its effect on normal hematopoiesis and MDS pathogenesis.

MODELLING MUTATIONS IN TRANSCRIPTION FACTORS AND EPIGENETIC MODIFIERS

RUNX1

The *RUNX1* gene, also known as *AML1* or *CBFA,* plays a key role in hematopoiesis, and is frequently mutated in MDS, *de novo* AML and secondary AML[102,103]. The vast majority of *RUNX1* mutations are located in the Runt homology domain (RHD) which mediates binding to DNA and core binding factor beta, although mutations in the C-terminus outside the RHD have also been reported[104].

Watanabe-Okochi *et al*[105] developed a *RUNX1* mouse model using retroviral constructs based on two types of *RUNX1* mutations identified in patients. AML1-D171N (hereafter D171N) has a point mutation in the RHD resulting in the loss of its DNA binding site, while the AML1-S291fsX300 (hereafter S291fs) has a frameshift mutation outside the RHD that results in C-terminal truncation, leading to the loss of transactivation potential but increased DNA-binding ability. Both are dominant negative forms, with the latter being more potent than the former[106,107].

Both D171N and S291fs mice developed macrocytosis, multi-lineage dysplasia, progressive cytopenias in a normal or hypercellular bone marrow, and transformation to leukemia in 4 to 13 mo. However, they displayed quite distinct phenotype and disease kinetics. D171N mice had a more proliferative phenotype with leukocytosis due to increased myelopoiesis, more prominent granulocytic dysplasia, accompanied by marked hepatosplenomegaly, and a higher percentage of blasts. In contrast, S291fs developed pancytopenia with a more marked erythroid dysplasia. This study showed that different mutations within the same gene could induce heterogeneous disease with different biological outcomes. This may be explained in part by the structural and functional differences between the mutants.

Of note, a fraction of the D171N mice had the *Evi1* locus as the retroviral integration site. This reduced the latency of leukaemic transformation to 3 to 5 mo, hence, providing evidence that *Evi1* co-operates with mutant RUNX1 to facilitate disease progression.

Ten-eleven-translocation-2

Ten-eleven-translocation-2 (*TET2*) belongs to a 3-member family of genes (*TET1-TET3*). It encodes a α-ketoglutarate-dependent enzyme that catalyzes the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethyl cytosine (5-hmC), which is the first step of active demethylation. It is frequently mutated in myeloid malignancies (up to 30% of MDS)[108,109].

Loss of *TET2* leads to a reduction in the amount of 5-hmC, and this has been demonstrated in samples of patients with myeloid malignancies, suggesting that *TET2* acts as a tumour suppressor gene[110]. Indeed, several groups have reported that the loss of *TET2* resulted in deregulated self-renewal of hematopoietic stem cells and the development of CMML-like disease[111-114]. It should also be noted that mice that were hypomorphic or heterozygous for the *TET2* allele showed similar phenotypes, suggesting a haploinsufficiency effect of *TET2* in the development of hematopoietic malignancies.

Recently, Muto *et al*[115] described mice hypomorphic for *TET2* as a mouse model of CMML and MDS. In that study, *TET2* gene trap mice ($TET2^{KD/KD}$) were engineered to express approximately 20% of the *TET2* mRNA of WT mice. $TET2^{KD/KD}$ mice developed overt features of myeloid malignancy after about 11 mo. Whilst the majority had features of CMML, 3 out of 13 mice developed MDS with pancytopenia, granulocytic dysplasia, and increased erythroid apoptosis.

Comparable to the *NHD13* model, $TET2^{KD/KD}$ mice

that developed CMML or MDS showed skewing in their HSPC compartment. These mice had a greater proportion of Lineage$^-$, Sca-1$^+$, c-kit$^+$ (LKS$^+$) cells in their bone marrow. Interestingly, mice with CMML had a greater proportion of CMP and granulocyte-macrophage progenitors (GMP) in comparison to mice with MDS who had a greater proportion of megakaryocyte/erythroid progenitors (MEP).

Of note, both mice with CMML and MDS developed splenomegaly, with further analysis showing marked increases in LKS$^+$ cells and disruption of the splenic architecture due to extramedullary hematopoiesis. It should be noted that although splenomegaly in association with CMML is well documented clinically, splenomegaly does not tend to feature in MDS patients. This can be explained by the hematopoietically active role of the spleen in the lifespan of the mice, but not in humans. As such, splenomegaly can be seen in MDS mice in the context of a compensatory response to underlying erythroid changes.

This study showed that alterations in TET2 expression resulted in two distinct phenotypes, CMML and MDS, after a considerable latency. Recurrent somatic *TET2* mutations have been identified in normal, elderly individuals with acquired clonal hematopoiesis without overt clinical manifestations[16]. In line with this, findings from this mouse study support a role for *TET2* mutations as early, founder or initiating mutations in myeloid malignancies with later, acquisition of additional, co-operating mutations required to bring about overt disease. The distinct phenotype observed, whether predominantly myelodysplastic, myeloproliferative or myelodysplastic/myeloproliferative may be determined by the nature of the secondary mutations.

Additional sex combs-like 1

Additional sex combs-like 1 (*ASXL1*) plays an important role in regulating *Hox* genes through its interaction with the polycomb group of proteins[116,117]. *ASXL1* mutations are reported in approximately 15%-20% of MDS patients[47,49,118]. They are usually heterozygous with most mutations located in the 5′ region of the last exon (exon 12), resulting in the expression of a truncated ASXL1 protein[119]. *ASXL1* mutations are generally subclonal, indicating that they are acquired later in the course of the disease. They have been reported to promote leukaemic transformation. and their presence is an independent predictor of adverse prognosis in MDS[120,121].

There are two mutated *ASXL1* mouse models of MDS in the literature[122,123]. In the first study, Inoue *et al*[122] developed retroviral constructs with a C-terminal-truncating *ASXL1* mutation, FLAG-*ASXL1*-MT1 and FLAG-*ASXL1*-MT2 (collectively termed *ASXL1*-MTs) derived from patients with MDS harbouring mutated genes of 1934dupG;G646WfsX12 and 1900-1922del;E635RfsX15 respectively. GFP-positive cells of *ASXL1*-MTs mice showed preferential myelopoiesis in the marrow at the expense of reduced B-lymphopoiesis at 6 mo post transplantation. At approximately 12 mo, mutant mice developed features of MDS with display of pancytopenia, multi-lineage dysplasia and impaired myeloid differentiation. Additionally, some of the secondary transplant recipients progressed to secondary leukemia. Gene expression profiles of hematopoietic cells from mice that developed MDS showed de-repression of homeobox a9 (Hoxa9) through inhibition of polycomb repressive complex 2 - mediated methylation of histone H3K27. Moreover, the *ASXL1* mutation led to upregulation of Mir125a and subsequent suppression of C-type lectin domain family 5, member a (Clec5a), which is involved in myeloid differentiation. Thus, this study identified an ASXL1-MT-Hoxa9-Mir-125a-Clec5a axis that is critical for *ASXL1*-mediated MDS pathogenesis.

In another study, Wang *et al*[123] constitutively deleted *Asxl1* (*Asxl1*$^{-/-}$), which resulted in significant embryonic lethality. Surviving *Asxl1*$^{-/-}$ mice showed profound developmental abnormalities that included dwarfism and anopthalmia. Perinatal mortality was high; 78% of mice died within 24 h of birth and no mice lived longer than 42 d. Hematologically, the surviving mice displayed features of MDS with multilineage cytopenia and dysplastic neutrophils in the peripheral blood. In the bone marrow, *Asxl1*$^{-/-}$ mice showed normal to increased bone marrow cellularity, with accompanying myeloid hyperplasia and reduced erythroid precursors. Moreover, *Asxl1*$^{-/-}$ mice had reduced LKS$^+$ cells and altered myeloid progenitors with increased GMP and reduced MEP, accompanied by increased apoptosis in the bone marrow.

Subsequently, the generation of heterozygous *Asxl1* mutation (*Asxl1*$^{+/-}$) mice showed that haploinsufficiency was sufficient for the development of MDS. These mice generally had a milder MDS phenotype, with some developing a phenotype that was more reminiscent of CMML.

Taken together, these two studies demonstrated that *ASXL1* mutation or deletion gives rise to MDS phenotypes, and suggest a tumour suppressor function for *ASXL1* in hematopoiesis.

TERT

Telomere shortening or dysfunction has been linked to advancing age, however, its direct role in causing MDS is unclear. Colla *et al*[124] recently engineered an inducible telomerase mouse model to study the chronic physiological DNA damage in the hematopoietic system. *TERT*^{ER}, a telomerase reverse transcriptase-estrogen receptor fusion protein was used, and inter-generational crosses of *TERT*$^{ER/ER}$ mice was carried out to elicit progressive telomere erosion. By the fourth and fifth generations (G4/G5), telomere dysfunction with attendant DNA damage signalling and severe tissue degeneration was evident[125].

The G4/G5 *TERT*$^{ER/ER}$ mice displayed characteristics of MDS as early as 3 mo. They demonstrated significant cytopenias including anemia, reduced lymphopoiesis with accompanying hypercellular bone marrow, increa-

sed myeloid-to-erythroid ratio, increased apoptosis and multi-lineage dysplasia[124]. Moreover, approximately 5% of the aged G4/G5 *TERT*$^{ER/ER}$ mice progressed to AML.

In the hematopoietic progenitor compartment, G4/G5 *TERT*$^{ER/ER}$ mice showed a significant increase in GMP, accompanied by loss or markedly reduced MEP and CMP that were not attributed to an increase in apoptosis. Further analysis showed a preferential accumulation of γ-H2AX and 53BP1 DNA damage foci in the CMP subpopulation, but not in GMP or MEP. Of note, tamoxifen induction of *TERT* at G4/G5 stage was able to restore telomeres, ceased DNA damage signalling and reversed the degenerative tissue phenotype seen.

Subsequently, long-term HSC isolated from 3-mo-old G0 or G4/G5 mice were transplantable into wild-type congenic recipients[124]. Transplant recipients developed a more severe phenotype, with skewed myeloid differentiation, trilineage dysplasia and an excess of blasts. Notably, one of the six mice transplanted with G5 HSC progressed to AML. Defective CMP differentiation suggested from *in vivo* studies was confirmed using *in vitro* methylcellulose clonogenic assays, which showed a profound impairment of myeloid differentiation with preferential granulo-monocytic commitment at the expense of the erythroid lineage.

At a molecular level, the defect in CMP differentiation was found to be related to decreased expression of genes involved in the 3'mRNA splicing or processing genes, resulting in abnormal RNA splicing[124]. It was noted that 40% of the aberrant splicing events in *TERT*$^{ER/ER}$ cells resulted in exon skipping, and a higher proportion of in exon retention. Moreover, RNA-sequencing analyses of *TERT*$^{ER/ER}$ CMP cells identified aberrantly spliced genes to be associated with various pathways linked to MDS pathogenesis including DNA repair, chromatin remodeling and histone modification. These gene sets were also enriched in CMP of mice and patients with *SRSF2* mutations.

This report provides the first evidence linking telomere dysfunction to reduced expression of splicing factors, which consequently drives abnormal myeloid differentiation.

CONCLUSION

At present, we have an expanding list of MDS mouse models, most of which displayed a range of typical features of MDS including cytopenias, dysplasia, ineffective hematopoiesis, and some of which also have the ability to transform to leukemia. The bone marrow environment has a distinct role in MDS, as highlighted by the ongoing challenges faced in xenografting MDS cells into the murine system.

Several candidate genes used to model MDS have demonstrated their role in the pathogenesis of MDS, and in some cases, also revealed other collaborating mutations leading to leukaemic transformation. Additionally, the impact of specific recurrent mutations within a single gene has also begun to be elucidated. More recently, the therapeutic potential and the mechanism of action of lenalidomide has also been explored using a del(5q) mouse model, and has certainly enhanced our understanding of this disease with further implications in the clinical context.

In this era of flourishing genetic medicine, there is no doubt that existing and emerging mouse models will continue to be valuable tools in improving our insights into this disease. The availability of the novel CRISPR-Cas9 genomic editing system is likely to hasten the generation of more models, and importantly, the engineering of more complex models that better reflects the disease heterogeneity.

It should be noted that the utility of mouse models in the study of MDS can only be optimized by a careful and systematic approach to their characterization, including the distinction between features that are common to a variety of myeloid malignancies and those that are unique to MDS. Drawing similarities to humans, a diagnosis of MDS is difficult to conclude without the triad of cytopenia, dysplasia and absence of AML (< 20% blasts). Secondarily, evidence of increased apoptosis in the marrow, which is characteristic of MDS, will make the diagnosis more convincing.

To make a diagnosis of MDS, close examination of various haematopoietic samples is essential. The normal ranges of different cellular compositions within the different haematopoietic compartments in a normal mouse should first be appreciated, and this was very well-illustrated and described by Yang *et al*[126]. Ideally, morphological assessments should be carried out by an experienced pathologist.

When mice are monitored for features of MDS, peripheral blood sampling should be examined over various time points. The presence of peripheral blood cytopenia that is persistent over time may point to the development of MDS, particularly if this is accompanied by dysplastic changes morphologically. Anaemia may be normocytic or macrocytic, the latter clearly more convincing of MDS. However, peripheral blood assessments alone are insufficient to confirm MDS, and a thorough cytological and histological examination of the bone marrow and spleen is required[59,60]. Additional examination and histological assessment of other tissues may also be required especially if AML is suspected.

Cytological assessments for MDS should include the enumeration of marrow and spleen cellularity, the review of cytopsin preparations for dysplastic features and calculation of myeloid to erythroid ratio. This can be further complemented by immunophenotypic analysis of the various mature and immature cellular populations within bone marrow, including the haematopoietic stem and progenitor compartments. Perls Prussian blue staining should also be carried out on cytospins preparation to assess for the presence of ringed sideroblasts. Ringed sideroblasts are rare in mice, but would be pathognomonic of MDS if present. Histological examination of the bone marrow and spleen is useful to confirm tissue cellularity, and importantly, it provides

better morphological evaluation of megakaryocytes, which can be underrepresented in cytospins preparations.

Finally, the understanding of the MDS initiating cell, and mechanisms responsible for leukemic transformation are some of the major questions that remained to be answered in MDS. It is hopeful that new mouse models created will shed more light on the functional interplay amongst the various genetic mutations present. An ultimate goal of this area of research is to use animal models to facilitate the development of new therapeutics in MDS and improve clinical outcomes.

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MINIREVIEWS

Clinical approach to diarrheal disorders in allogeneic hematopoietic stem cell transplant recipients

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Abstract

Diarrhea is a common complication of allogeneic hematopoietic stem cell transplant (HSCT), with an average incidence of approximately 40%-50%. A wide variety of etiologies can contribute to diarrhea in HSCT patients, including medication-induced mucosal inflammation, infections, graft-*vs*-host disease and cord colitis syndrome in umbilical cord blood transplant. Clinical manifestations can vary from isolated diarrheal episodes, to other organ involvement including pneumonia or myocarditis, and rarely multiorgan failure. The approach for diagnosis of diarrheal disorders in HSCT patients depends on the most likely cause. Given the risk of life-threatening conditions, the development of clinically significant diarrhea requires prompt evaluation, supportive care and specific therapy, as indicated. Serious metabolic and nutritional disturbances can happen in HSCT patients, and may even lead to mortality. In this review, we aim to provide a practical approach to diagnosis and management of diarrhea in the post-transplant period.

Key words: Diarrhea; Medication-induced diarrhea; Allogeneic hematopoietic stem cell transplant; Enteric infection; Graft-*vs*-host disease

Core tip: Diarrhea is a common complication following allogeneic hematopoietic stem cell transplant. However, there is no recent review dedicated to guiding clinicians about the different causes and their management. Our objective is to conduct a thorough review of literature to provide a working schema for the busy clinician on

evaluation and management of diarrhea in this special population.

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INTRODUCTION

Hematopoietic stem cell transplant (HSCT) is increasingly used to treat a variety of hematologic disorders. Worldwide, more than 50000 HSCTs are performed every year; approximately 40% of these HSCTs are allogeneic HSCT[1]. Given the increased volume of HSCT procedures, the number of HSCT-associated toxicities is expected to increase. Gastrointestinal complications are common in HSCT recipients and significantly contribute to HSCT-related morbidity and mortality. A wide variety of gastrointestinal complications are reported including nausea, vomiting, oropharyngeal mucositis, dysphagia, diarrhea, gastrointestinal bleeding and graft-*vs*-host disease (GVHD) of the gastrointestinal tract. These manifestations are seen in more than two-third of patients undergoing HSCT[2].

Diarrhea is a common complication of HSCT, with an average incidence of approximately 40%-50%, with a higher occurrence within the first several weeks post-transplant[3,4]. High-dose chemotherapy and radiotherapy used for myeloablation, without any other identifiable etiology, can alone cause diarrhea in up to 50% of patients[2]. Here, we will review a practical approach to diagnosis and management of diarrhea in the post-transplant period.

ETIOPATHOGENESIS

A wide variety of etiologies can contribute to diarrhea in HSCT patients, including mucosal inflammation caused by medications used for myeloablation or immunosuppression, infections, GVHD or other causes seen in non-transplant patients (Tables 1 and 2)[2-7]. Apart from conditioning regimen and immunosuppressant (*e.g.,* mycophenolate mofetil or tacrolimus), antibiotics, proton pump inhibitors, promotility agents (*e.g.,* metoclopramide), and magnesium salts are common causes of medication-induced diarrhea. Infection accounts for 5%-10% of diarrhea in adults HSCT patients[2-7]. Among different infectious etiologies, viruses are the most common pathogens and include cytomegalovirus (CMV), herpes simplex virus (HSV), Ebstein-Barr virus, adenovirus, norovirus and enteric viruses such as rotavirus, coxsackie, and echo virus. Clinical manifestations can vary from isolated diarrheal episodes, to other organ involvement including pneumonia or myocarditis, and rarely multiorgan failure. Clostridium difficile remains to be an important cause of antibiotic-associated diarrhea[3]. Its occurrence is related to the exposure to multiple antibiotics, antineoplastic agents, prolonged hospitalizations, and extended period of reduced host immunity that can lead to disruption of normal intestinal epithelium. Clostridium difficile may possibly contribute to an increased risk of gastrointestinal GVHD as well by providing the antigenic substrate and adding to the mucosal damage by activated donor T cells that release pro-inflammatory cytokines that drives the immune response towards GVHD[8]. Neutropenic enterocolitis, characterized by cecal involvement and occasionally ascending colon, can result from polymicrobial infection following a combination of mucosal cytotoxic injury, impaired host defense and profound neutropenia. The interaction of pro-inflammatory mediators present in the lumen with innate immune system in the intestinal submucosa leads to the release of pro-inflammatory cytokines resulting in epithelial cell apoptosis and increased mucosal permeability culminating into the syndrome of neutropenic enterocolitis. Microscopically there is a minimal inflammatory exudate along with submucosal edema and hemorrhagic necrosis. Thus, without the presence of any inflammatory response, different bacterial and/or fungal organisms including gram-negative bacilli, gram-positive cocci, anaerobes (*e.g., Clostridium septicum*) and *Candida* species can readily translocate through the bowel wall causing bloodstream infection[9].

Acute GVHD, neutropenic enterocolitis/typhlitis or cytomegalovirus-colitis are among the serious causes of diarrhea and can cause significant morbidity, prolonged hospitalization and increased non-relapse mortality[9-11]. Any moderate to severe diarrhea, regardless of its cause, particularly when associated with vomiting, can contribute to fluid and electrolyte losses, malnutrition, requirement for parenteral nutrition, deconditioning and slow recovery from the effects of conditioning regimen and HSCT.

CLINICAL FEATURES

Medication-induced diarrheal disorders are watery and usually not associated with bloody stool or significant abdominal pain[12]; however, nausea, vomiting, anorexia and mild abdominal cramps are not uncommon in HSCT recipients. Clinical manifestations in viral infections can vary from isolated diarrheal episodes, to other organ involvement including pneumonia or myocarditis, and rarely multiorgan failure[10]. *Clostridium difficile* (*C. difficile*) infection causes multiple episodes of watery diarrhea, associated with abdominal cramp, nausea, low-grade fever and leukocytosis[8]; however, it can also cause severe disease with mortality rate reaching up to 20%, particularly in patients who were previously treated with linezolid[13]. Neutropenic enterocolitis presents with abdominal pain, fever, bloody or watery diarrhea and occasionally nausea and vomiting. The

Table 1 Common causes of diarrhea in hematopoietic stem cell transplant recipients

Etiology	Time period	Percentage of diarrheal episodes	Tests	Management	Comments
Acute GVHD	Early post engraftment	40%-60%, particularly after engraftment	Colonoscopy and biopsy	High-dose prednisone; if no response, other immunomodulators, and extracorporeal photopheresis	Steroid-refractory gut acute GVHD can be fatal
Conditioning regimen, without other etiology	Within 5-7 d after chemotherapy	50%	No-specific tests, other etiologies need to be ruled out	Supportive care	
Medications	During any time, usually within few weeks after initiation	Variable	No-specific tests, other etiologies need to be ruled out	Supportive care, medication withdrawal if possible	Usually diarrhea stops after cessation of the offending medication
Infections	Pre-engraftment for Clostridium difficile infection and typhlitis; early post-engraftment for enterovirus, adenovirus, CMV colitis	5%-10%	Microbiologic, molecular or pathologic tests; CT, CTE, or MRE; colonoscopy with biopsies	Supportive care if viral, antibiotics if bacterial, antifungal therapy if fungal	Neutropenic enterocolitis and CMV colitis can be life threating in severe cases
Cord colitis	Late post-engraftment	10% of cord transplant	Negative cultures and a colon biopsy demonstrating chronic active colitis	Metronidazole	Only occurs in recipients of umbilical cord blood transplant

Also consider other causes unrelated to transplant such as malabsorption syndrome including pancreatic exocrine insufficiency, adrenal insufficiency, lactose intolerance, or other malabsorption syndromes[2-7]. CMV: Cytomegalovirus; CT: Computerized tomography; CTE: Computerized tomography enterography; GVHD: Graft-*vs*-host disease; MRE: Magnetic resonance enterography.

Table 2 Most common medications and infectious etiologies causing diarrhea in hematopoietic stem cell transplant recipients

Categories	Agents
Medications	Conditioning regimen, antibiotics, mycophenolate mofetil, tacrolimus, proton pump inhibitors, promotility agents, magnesium salts
Viral infection	Cytomegalovirus, herpes simplex virus, Ebstein-barr virus, adenovirus, norovirus and other enteroviruses
Bacterial infection	*Campylobacter*, *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia*, *Vibrio cholera*, *Clostridium difficile*, other enteric pathogens
Others	Fungal, *e.g.*, candida, parasitic, *e.g.*, Cryptosporidium and *Mycobacterial* infections
Mixed infection	Neutropenic enterocolitis

abdominal pain can be generalized or localized usually in the right lower quadrant. In severe cases, patients may develop acute abdomen with peritonitis, often indicative of complications such as perforation or abscess formation[9].

Acute GVHD is among the most common cause of diarrhea in HSCT patients, accounting for one-third of cases in some studies, especially if diarrhea persists beyond 3 wk of a HSCT[3,4]. Symptoms vary from watery to bloody diarrhea, usually high volume, associated with abdominal pain or cramping. The bleeding can be serious to require blood transfusion. If upper gastrointestinal tract is involved, patients may develop nausea, vomiting and anorexia. Other features of GVHD such as skin rash may also be present[11].

Cord colitis syndrome occurs only in recipients of umbilical cord blood transplant, with an occurrence rate of 10% in such patients. It manifests as watery diarrhea, presenting at about 4 mo after the transplant, and persisting for more than a week. Fever and weight loss are not uncommon, and the majority of patients require hospitalization. Colonoscopy usually reveals erythematous mucosa with or without ulcerations, with occasional granuloma formation[6]. It responds to metronidazole[6] despite negative viral and bacterial cultures, and is felt to be related to infection with bradyrhizobium enteric[14].

EVALUATION AND DIAGNOSIS

The approach for diagnosis of diarrheal disorders in HSCT patients depends on the most likely cause. Given the risk of life-threatening conditions, the development of clinically significant diarrhea requires prompt evaluation, supportive care and specific therapy, as indicated. A thorough history and review of medications may provide information regarding possible medication-induced diarrhea. Stool analysis including viral culture and *C. difficile* toxin assay or polymerase chain reaction (PCR) should be performed. Monitoring of renal function, electrolyte and nutritional status are important. Timing of onset of diarrhea may provide an idea about potential causes.

Pre-engraftment period

Medications including conditioning regimen, calcineurin inhibitors, oral magnesium and prophylactic antimicrobials are frequently used in HSCT recipients, hence

important causes of diarrhea[12]. *C. difficile* is another common cause during this phase and is seen in up to 10%-20% of the patients[8,13,15]. Detection of *C. difficile* toxin or the toxigenic *C. difficile* organism in the stool can be achieved with PCR, enzyme immunoassay for *C. difficile* toxins A/B or *C. difficile* glutamate dehydrogenase[16]. Although non-diagnostic, the classic computed tomography (CT) findings include pancolitis with marked colonic wall thickening (11-15 mm) and wall nodularity, most frequently involving sigmoid colon and the rectum[17,18]. Contrast-enhanced CT scan of the abdomen may show a characteristic but non-specific finding of "target sign" or "double halo sign" due to submucosal edema and mucosal enhancement. Colonic edema may also result in "accordion sign" due to thickening of the haustral folds[19]. Other non-specific imaging features include pericolic fat stranding and ascites. Colonoscopy, performed to rule out other diagnosis, may demonstrate pseudomembrane formation, apoptosis and nonspecific ulceration. However, the absence of pseudomembrane does not rule out the *C. difficile* infection in HSCT patients[20,21].

Neutropenic enterocolitis is a common cause of diarrhea during this phase[9]. Initial workup for suspected typhlitis includes *C. difficile* toxin assays, stool and blood cultures. All patients with suspected neutropenic enterocolitis should have CT scan of the abdomen with intravenous and oral contrast in the absence of any contraindication for contrast use. CT scan of the abdomen has a lower false-negative rate (15%) than ultrasound (23%) or plain radiographs of the abdomen (48%)[22]. CT findings are usually less severe than that seen in pseudomembranous colitis and include bowel wall thickening (7 mm on average, range 4-15 mm), especially in the ileocecal area[23]. Pneumatosis intestinalis, seen in up to 20% of cases[23], is usually not seen in other non-vascular causes of colitis or GVHD[17]. Other non-specific findings include mesenteric stranding (51%), bowel dilatation (38%), and mucosal enhancement (28%)[23]. Plain films of the abdomen are nonspecific but may show fluid-filled, distended cecum with dilated adjacent small bowel loops, thumb-printing or localized pneumatosis intestinalis[24].

Early post-engraftment period

Medications and *C. difficile* infection are common causes of diarrhea during this period as well. Additionally, viral infection (*e.g.,* enteric virus, adenovirus and CMV virus) and acute GVHD of gut are important etiologies. Enteric virus infections (*e.g.,* coxsackie A, rotavirus or norovirus) can cause prolonged gastroenteritis among HSCT recipients. Nevertheless, isolation of virus in a stool specimen, even in cases of diarrhea, may be the result of viral shedding and not be related to intestinal infection[4]. The specific diagnosis of adenovirus infection is also challenging and may require the use of multiple diagnostic tests. Viral culture, direct antigen assays, or PCR of upper nasopharyngeal, throat, urine, stool or rectal samples may detect viral shedding. Results should be correlated with the clinical picture. Quantitative PCR of blood may be helpful to establish the diagnosis, evaluate the risk of dissemination, monitor response to antiviral therapy and determine prognosis[25-28].

CMV colitis is an important differential of diarrheal disorder in HSCT patients. The diagnosis is challenging and may be complicated by the presence of concomitant gastrointestinal GVHD. The final diagnosis of CMV is based on the presence of any positive microbiological, molecular or pathological tests identifying CMV, and a response to treatment. Diagnostic tests for active CMV disease include CMV phosphoprotein 65 antigenemia assay and quantitative nucleic acid testing, which may be used to establish diagnosis, determine a need to initiate pre-emptive therapy, and monitor response to therapy[29,30]. Viral culture of blood, urine or tissue specimens can be slow and expensive, and is also less sensitive and specific than molecular diagnostic assays. Culture of gastrointestinal tissue for the diagnosis of tissue-invasive disease can be an option when both antigenemia and PCR testing on blood are negative[31]. Sigmoidoscopy with biopsy appears to have equivalent diagnostic yield for CMV colitis, compared to colonoscopy[32]. Typical histopathology finding includes the presence of viral nuclear inclusions but the presence of gastrointestinal gland apoptosis without viral inclusions does not rule out CMV involvement[33]. If a CT scan is done, findings usually include ascending colon wall thickening with an average thickness of 15 mm[34]. The most characteristic feature is mural edema with deep ulcerations, likely due to occlusive vasculitic process, with or without small bowel involvement, surrounding fat stranding and ascites[35].

Acute GVHD of gut is the most frequent cause of diarrhea during the early post-engraftment period, and most commonly occurs within the first few months after HSCT or following a reduction in immunosuppression. The presence of a maculopapular skin rash or otherwise unexplained elevated serum bilirubin or alkaline phosphatase within the first 100 d of HSCT may support the diagnosis. Most but not all cases of acute GVHD of lower gastrointestinal tract can be diagnosed by rectal biopsy[36,37]. Hence, a negative rectal biopsy in patients with a clinical suspicion of acute GVHD requires evaluation with colonoscopy. Additionally, multiple colonic biopsies and pathologic evaluation of tissue are necessary for the diagnosis even when mucosal lining of colon appears normal[37]. Epithelial cell apoptosis, particularly involving cryptic cells, is the characteristic histological feature observed in patients with acute gastrointestinal GVHD[38]. A study has indicated that the presence of 6 or less apoptotic bodies per 10 contiguous crypts may not be predictive of gastrointestinal GVHD[39]. The presence of concurrent GVHD in another organ greatly increases the specificity of isolated crypt apoptosis as a diagnostic feature of gastrointestinal GVHD[21]. Epithelial cell apoptosis can be seen after the use of conditioning regimen but the changes are noted within the first 20 d, whereas acute GVHD is only seen

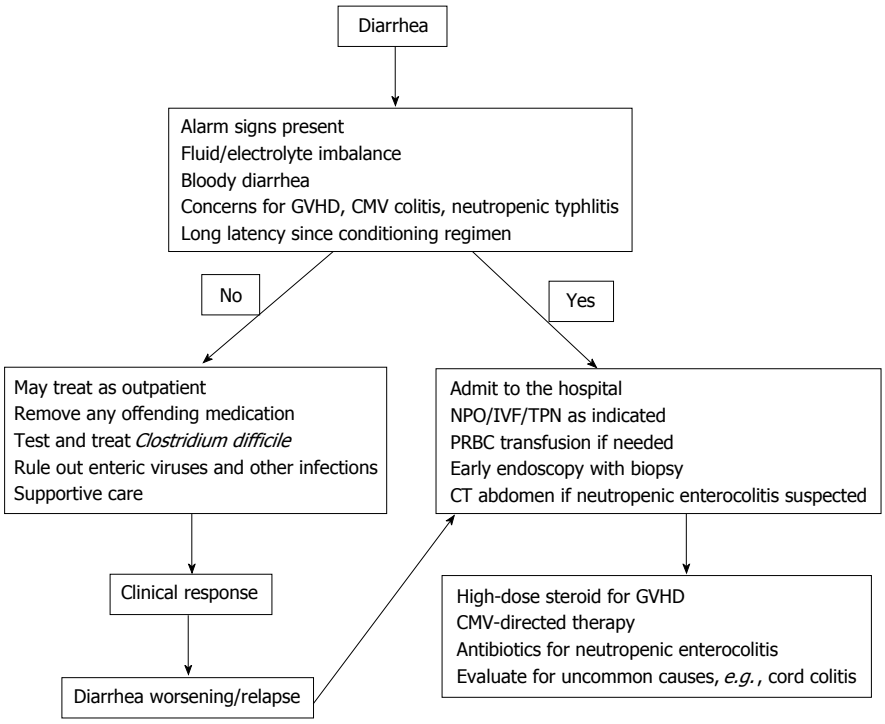

Figure 1 Algorithm for management of diarrhea in transplant recipients. CMV: Cytomegalovirus; CT: Computerized tomography; GVHD: Graft-*vs*-host disease; IVF: Intravenous fluids; NPO: Nil per os; PRBC: Packed red blood cells; TPN: Total parental nutrition.

after engraftment, which occurs after the second or third week following the transplant[40]. Other differentials that can cause gland epithelial apoptosis include drugs such as mycophenolate mofetil and proton pump inhibitors, and viruses such as adenovirus and CMV infection[38]. Radiologic findings are not diagnostic of GVHD. The most consistent CT finding is abnormal mucosal enhancement of the entire gastrointestinal tract, mostly affecting the small bowel. Other frequent features include dilated, fluid-filled bowel loops, submucosal edema with "target sign"[34]. However, a wall thickness of greater than 7 mm can help exclude GVHD[23].

Late post-engraftment period

Diarrhea is more likely to be related to aforementioned infections, but less commonly could be a manifestation of chronic GVHD, particularly if other features of chronic GVHD are also present. The chances of drug-induced diarrhea from mycophenolate, tacrolimus or prophylactic antimicrobial may be relatively less, unless the medications have been changed recently. Other causes of diarrhea, as observed in general population, including pancreatic insufficiency should also be considered. Adrenal insufficiency, possible because of steroid exposure for treatment of GVHD, may also result in gastrointestinal symptoms including diarrhea. If etiologies are not established, a colonoscopy and biopsy may be needed for diagnosis. No histological changes involving the colon are specific for chronic GVHD, but common features may include apoptotic epithelial cells and crypt drop out. These features, however, are also seen in patients with inflammatory bowel disease[38,41]. Cord colitis syndrome, exclusively seen in cord blood transplant recipients, may mimic chronic GVHD[42]. It is distinguished from other causes of diarrhea by negative viral and bacterial cultures, and a colon biopsy that demonstrates chronic active colitis, frequently with associated granulomas. A response to antibacterial treatment can be both diagnostic and therapeutic[6,14].

MANAGEMENT

Diarrhea in HSCT recipients can induce serious metabolic and nutritional disturbances and may even lead to mortality. Therefore, many HSCT patients with diarrhea may require inpatient admission for volume and electrolytes replacement. However, some patients may be treated as an outpatient with close follow up if they do not have alarming signs such as bloody diarrhea, significant abdominal pain, fever, inability to tolerate oral intake, hypovolemia, tachycardia or hypotension (Figure 1).

Supportive care

If infectious causes of diarrhea are ruled out, loperamide can be used at an initial dose of 4 mg orally, followed by 2 mg with each bowel movement as needed up to a total of 16 mg in 24 h. If diarrhea persists, atropine, diphenoxylate, bismuth subsalisylate, or tincture of opium may be added to the scheduled loperamide. Octreotide may be useful in select cases, particularly when it is started early on in GVHD[43,44]. Other supportive measures that may be used in moderate to severe cases include the use of cholestyramine, bowel rest,

hydration, electrolyte replacement and parenteral nutrition.

GVHD

High-dose steroid remains the preferred frontline therapy for both acute GVHD (1-2 mg/kg per day of prednisone) and chronic GVHD (1 mg/kg per day of prednisone) of gut. High-dose steroid is continued for 1-2 wk or until response, than it is tapered over next several weeks[11,45]. Patients with acute GVHD, who respond to high dose prednisone within the fifth day of therapy, have significantly improved outcomes, compared to non-responders[46]. Non-absorbable steroids such as budesonide can improve response, reduce steroid doses and prevent relapse of gastrointestinal GVHD following the tapering of prednisone[47]. Addition of any other agent to prednisone as a frontline therapy has not been shown to improve outcomes. For steroid-refractory patients, outcomes are poor but several therapy agents have been used including extracorporeal photopheresis, calcineurin inhibitors, sirolimus, etanercept, pentostatin, among others[11,45].

Infections

Identifying the offending organism is crucial in decisions about further treatment of infectious enterocolitis. Culture and susceptibility may guide therapy selection. *C. difficile* infection needs a prompt treatment, and clinicians should treat empirically in suspicious cases because of a high mortality rate. Treatment options include metronidazole, and vancomycin for complicated cases. Fidaxomicin has been recently approved by Food and Drug Administration, and can be considered in refractory cases. Fecal transplant is yet another treatment modality in recurrent/refractory cases[48]. Certain viral diarrhea with enteric viruses may be self-limited but require cautious monitoring; however, where specific agents are available, therapy should be instituted. Ganciclovir or valacyclovir is the therapy of choice for CMV, whereas HSV infection may be treated with acyclovir or valacyclovir for 2-3 wk. Foscarnet is the second-line therapy in both cases[49]. Although data regarding bacterial gastroenteritis are lacking in HSCT patients, empirical antibiotics are not usually indicated in the absence of clinically significant fevers, bloody stools, and evidence of invasive disease based on significant white blood cell or red blood cells in the stool. Trimethoprim/sulfamethoxazole has a good coverage for most of the enteric bacteria and may be used as the first line therapy. Quinolones also provide a good coverage for the enteric bacteria, but the concerns exist regarding an increased resistance to quinolones. Erythromycin is a good therapy option for campylobacter-related diarrhea[50].

Neutropenic enterocolitis

Treatment of neutropenic enterocolitis requires high level of care, as it is potentially life threatening. All patients should be treated as an inpatient with intravenous fluids, nasogastric tube placement for gastric decompression, and bowel rest. Anti-diarrheal should be avoided as it may aggravate the ileus and worsen the symptoms. Broad-spectrum antibiotics with good coverage against anaerobes and gram-negative rods including pseudomonas are recommended. Suggested empiric regimens include tazobactam-piperacillin, cefepime plus metronidazole, or carbapenem plus metronidazole. The therapy should be adjusted based on the culture results. A lack of response to broad-spectrum antibiotics within 72 h should raise the suspicion for fungal etiology. In such context, addition of antifungal therapy may have to be considered. Intravenous antibiotics can be switched to oral after 48 h of being afebrile and recovery of absolute neutrophil count to above 500/μL. However, antibiotics should be continued for additional 14 d[9,51,52]. Surgical exploration is largely avoided in patients with neutropenic enterocolitis, but may have to be considered in severe cases with suspected perforation or bowel necrosis. Other potential indications for surgical intervention may include clinical deterioration despite the appropriate treatment, or persistent bleeding[9,53].

Cord colitis syndrome

Antibiotic therapy with metronidazole alone or with quinolones for a total of 14 d is the mainstay of the treatment[6].

CONCLUSION

Diarrhea is common in HSCT recipients and accounts for significant morbidity and possibly mortality in these patients. The increasing use of HSCT procedures worldwide indicates that the diarrheal disorder in this patient population is likely to rise. Mediations, infections and GVHD are common causes of diarrhea in post-transplant period[2-7]. Medication history, stool analysis, culture, and *C. difficile* PCR should be routinely performed to establish the underlying cause. CT scan and colonoscopy with biopsies may be performed if the other tests are unrevealing and may help diagnose conditions such as neutropenic enterocolitis and GVHD[23,38,54]. In addition to supportive care such as hydration, monitoring and correction of electrolytes and acid-base balance, timely initiation of appropriate specific treatment is important to improve outcomes. Management often requires a specialized multidisciplinary transplant team with experienced transplant physicians, gastroenterologists, infection disease experts and nursing staff. Many HSCT recipients leave the transplant centers after initial hospitalization and follow-up with community oncologists, hence training of community oncologists and patient education in post-transplant issues are important. Frequent communication between community oncologists, patient/caregivers and transplant physicians can help expedite diagnosis and initiate early management. Certain diagnoses such as neutropenic enterocolitis, steroid-refractory acute GVHD and CMV

colitis are difficult to treat with current therapeutic modalities, hence further research to optimize therapy is needed.

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MINIREVIEWS

Advances and perspectives on cellular therapy in acquired bone marrow failure diseases

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Abstract

Acquired bone marrow failure diseases (ABMFD) are a class of hematopoietic stem cell diseases with a commonality of non-inherited disruption of hematopoiesis that results in pancytopenia. ABMFDs also are a group of heterogeneous diseases with different etiologies and treatment options. The three most common ABMFDs are aplastic anemia, myelodysplastic syndrome, and paroxysmal nocturnal hemoglobinuria. Stem cell transplantation is the only treatment that can cure these diseases. However, due to high therapy-related mortality, stem cell transplantation has rarely been used as a first line treatment in treating ABMFD. With the advance of personalized medicine and precision medicine, various novel cellular therapy strategies are in trial to increase the efficiency and efficacy of ABMFD treatment. This article aims to review current available stem cell transplantation protocols and promising cellular therapy research in treating ABMFD.

Key words: Bone marrow failure diseases; Aplastic anemia; Cellular therapy; Stem cell transplantation; Paroxysmal nocturnal hemoglobinuria; Myelodysplastic syndrome

Core tip: Stem cell transplantation is the only method can cure acquired bone marrow failure diseases (ABMFD). However, due to the high mortality rate of stem cell transplantation itself, this method is not usually used as the first line treatment for ABMFD. With

the advance of current cellular therapy technology, it is becoming possible to cure ABMFD without significant treatment related complications.

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INTRODUCTION

Acquired bone marrow failure diseases (ABMFD) are a group of rare hematologic disorders manifested by insufficient hematopoiesis to produce a sufficient amount of red blood cells, white blood cells, or thrombocytes. ABMFD can occur after exposure to viral infections, toxins, chemicals, or radiation. ABMFD includes aplastic anemia (AA), myelodysplastic syndrome (MDS), and paroxysmal nocturnal hemoglobinuria (PNH). Though the pathogenesis of these diseases is heterogeneous, the high similarity of their clinical manifestation and their bone marrow pathophysiological presentation makes them hard to distinguish from each other. ABMFD can be cured by stem cell transplantation. However, because of the high mortality rate of this therapy, stem cell transplantation has not usually been used as a first line treatment for ABMFD. Currently, there is a lack of literature that offers insight into ABMFD as a class of disorders. This review offers a comprehensive overview of many of the standard and novel treatment options.

CLINICAL PRESENTATION AND CURRENT MANAGEMENT OPTIONS

The common clinical presentations for AA, MDS and PNH are cytopenia in single or multiple hematological lineages, together with signs of impaired quality of life such as fatigue, dizziness, headache, shortness of breath, and other symptoms that are associated with prolonged anemia. The individual clinical presentations of AA, MDS and PNH are illustrated as Figure 1. Due to nearly indistinguishable clinical presentation, peripheral blood smear and bone marrow biopsies are used in the diagnosis of ABMFD.

MDS is the most common form of ABMFD, affecting around 15000 Americans each year[1]. The risk of MDS increases with age[2]: It typically affects people at age 60 years or older. In MDS, myeloid stem cell dysfunction in the bone marrow leads to ineffective hematopoiesis[3]. If left untreated, some of MDS can progress into acute myeloid leukemia. Cancer drugs such as chlorambucil, cyclophosphamide, doxorubicin, ifosfamide, mechlorethanmine, melphalan, procarbazine, and etoposide are associated with onset of treatment related MDS[3].

AA is the second most common form of ABMFD, with an incidence rate of 2.0/million to 7.4/million worldwide[4], and can be triggered by toxins, radiation, chemotherapy, viruses, medicines, autoimmune disorders, or pregnancy[4]. In AA, the bone marrow is injured and the hematopoiesis is interrupted. In most cases, AA is secondary to immune system dysfunction and subsequent premature turnover of hematopoietic cells. AA is commonly seen in young adults.

PNH is a rare hemolytic disease caused by complement system attack on cells with surface membrane glycosylphosphatidylinositol (GPI)-anchor protein deficiency. PNH affects roughly 6000 Americans each year. The clinical presentation of PNH includes hemolytic anemia, thrombosis in large blood vessels, and cytopenia or pancytopenia, depending on severity[5]. PNH appears in suddenly, but recurring episodes can be triggered by stress or physical exertion of the body. Attack on both hematopoietic cells and mature blood cells leads to formation of abnormal blood cells[6]. Abnormally weakened red blood cells will rupture. The ruptured red blood cells will release free hemoglobin that is then excreted through the kidney and stains the urine dark-colored.

TRANSFUSION THERAPY

Transfusion therapy is recommended as a part of supportive therapy for all ABMFDs[7,8]. The current transfusion guidelines suggests transfusion for those patients with platelet counts below 10×10^9/L (or $< 20 \times 10^9$/L in febrile patients), though the ultimate decision for transfusion should be based on the patient's overall clinical condition. Transfusions should be used cautiously because it can induce alloimmunization and autoimmunization that will complicate future treatments, such as hematopoietic cell transplantation (HCT).

Transfusion using blood from family members may induce sensitization against human leukocyte antigens (HLA) of potential HCT donors. The blood units should be carefully screened for common viruses (such as cytomegalovirus, human immunodeficiency virus, human T-lymphotropic retroviruses, hepatitis B and C, and West Nile virus), undergo leukocyte reduction, and be irradiated to avoid graft-*vs*-host disease (GVHD). Platelet transfusion is useful to prevent or stop thrombocytopenic bleeding. Platelet units are useful only for 3-7 d. Platelet shall be stored at room temperature to keep its activity, which, on the otherside, increases the risk of transfusion related infection. White blood cell transfusion is not highly recommended due to efficacy issue.

IMMUNOSUPPRESSIVE THERAPY

For AA and PNH, immunosuppressive therapy (IST) is a front line management to treat immune system dysfunction[9,10]. The complement system is a part of immune system that facilitates leukocytes and antibodies

Table 1 Advantages and disadvantages of hematopoietic stem cell transplantantion

Sources of stem cells for transplantation	Peripheral blood	Bone marrow	Cord blood
Advantages	Abundant supply Easy to collect and differentiate No surgical procedure Short recovery period Fastest engraftment Low rates of morbidity and mortality	Abundant supply Easy to storage Relatively fast engraftment Autologous cells are immune compatible	Rapid procedure Less GVHD Tolerance of HLA-mismatching
Disadvantages	High risk of GVHD Requirement of close HLA-matching	Surgical procedure Long recovery period High risk of GVHD Requirement of close HLA-matching	Limited number of stem cells Difficult to grow and differentiate Slow engraftment Tissue rejection

GVHD: Graft-*vs*-host disease; HLA: Human leukocyte antigens.

in removing pathogens. However, the over activated complement system attacking GPI-anchor protein deficient stem cells in bone marrow is the mechanism of PNH[11,12]. Different types of immunosuppressive agents, such as antithymocyte globulin (ATG), cyclosporine-A (CSA), or various anti-complement anti-bodies or complement blockers, are used with high degree of response and survival[10,13,14].

ANDROGENS

Androgens (naturally occurring male hormones) have long been used as supportive treatment for many forms of anemia, including ABMFD[10]. Either injection of androgen (testosterone) or giving medications to increase endogenous androgen production are the common approaches to increase serum androgen level. The elevated androgen levels in patient's body may have gender-specific side effects: Men may experience enlargement of breasts or prostate, while women may experience facial hair growth, development of muscles, deepening of voice, or enlargement of the clitoris. Other side effects such as acne, jaundice due to increases in liver enzymes, and liver damage may occur. Due to the wide range of side effects, androgen therapy is limited and is typically used in combination with blood transfusions.

CELLULAR THERAPIES

Hematopoietic stem cell transplantation (HSCT) is the process of treating with a conditioning regimen followed by infusion of a healthy donor's mononuclear cells rich in hematopoietic stem cells and progenitor cells. In general, HSCT can be autologous (obtained from the patient's own cells), syngeneic (obtained from the patient's identical twin), or allogeneic (obtained from another individual); however, autologous HSCT is usually not a choice for ABMFD because the patient's lack of hematopoietic stem cells. The hematopoietic stem cells can be derived from either bone marrow, peripheral blood, or umbilical cord blood. The advantages and disadvantages of each type of hematopoietic stem cell transplantation are shown in Table 1. GVHD is one of the most common and serious complications. The risk and the severity of GVHD are largely related with the degree of HLA tissue type match between the donor and the recipient. Typically, a sibling has a 25% probability of being a perfect match for the recipient's eight major HLA antigens. The chance of finding an unrelated match ranges from 10% for some minority groups, to around 60%-70% for Caucasians in the United States.

Quite often, ABMFD occurs in patients that receive high doses of radiation therapy and/or chemotherapy. HSCT is often used, following cancer treatments, to facilitate recovery from high doses of radiation therapy and/or chemotherapy by replacing damaged or destroyed stem cells in the bone marrow and restoring hematopoiesis. HSCT for ABMFD has showed promising results[15].

PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

Peripheral blood stem cell transplantation (PBSCT) involves harvesting stem cells from the peripheral blood cells (peripheral blood is composed of erythrocytes, leukocytes and platelets) of the donor[16]. Before harvesting, donors are usually injected with granulocyte colony-stimulating factor to promote stem cell growth and release into the peripheral blood[17]. Currently, PBSCT is the most commonly performed HSCT due to easy access to peripheral blood stem cells and quick donor peripheral blood cell recovery[16].

BONE MARROW STEM CELL TRANSPLANTATION

Harvesting bone marrow stem cells is particularly complex procedure, compared to harvesting peripheral blood and umbilical cord blood. The donor must be given a general anesthetic and placed in an operation room. During the procedure, an aspiration needle is inserted at multiple points of the iliac crest region to collect approximately one liter (10-15 mL/kg) of bone

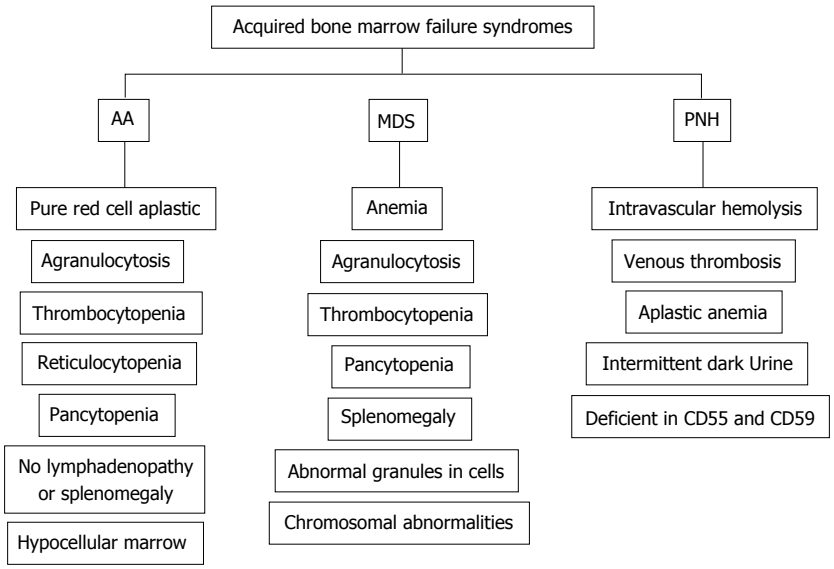

Figure 1 Typical clinical presentation of aplastic anemia, myelodysplastic syndrome and paroxysmal nocturnal hemoglobinuria. AA: Aplastic anemia; MDS: Myelodysplastic syndrome; PNH: Paroxysmal nocturnal hemoglobinuria.

marrow targeting a harvest of 2-4 × 10^8 nucleated cells per kilogram of recipient weight. The marrow is then filtered prior to infusion into the recipient. In the past, bone marrow stem cell transplantation was the only option available for HSCT, but due to the many obstacles in harvesting and health risks to the donor, other HSCT sources are becoming more frequently used. However, bone marrow stem cell transplantation is still a preferred option for ABMFD partially due to fewer amounts of lymphocytes in bone marrow reducing the risk and intensity of GVHD.

UMBILICAL CORD BLOOD TRANSPLANTATION

Umbilical cord blood collections are typically obtained from allogeneic, unrelated donors[18]. Cord blood is harvested from the leftover blood of the placenta and umbilical cord after a birth. The hematopoietic stem cells are filtered from the cord blood and kept frozen in storage. Total cord blood stem cell content is usually less than that obtained from peripheral blood or bone marrow, but the cord blood stem cells have higher hematopoietic potential and are able to produce more blood per cell than their counterparts. Due to the lesser quantity of cord blood stem cells, this type of transplantation is given to children or adults of smaller stature. There does not seem to be a strong association between HLA matching and acquiring GVHD and only one-third of patients can find a HLA-identical donor[19,20]. Thus, cord blood transplantation is beneficial for patients that cannot find an acceptable donor based on their HLA loci[21].

COMPARING IST AND HSCT

As of yet, there have been no clinical trials that have compared IST and HSCT. However, many cohort studies have been completed to analyze overall survival, quality of life and failure-free survival. Survival using HSCT is highly dependent upon the age of patients and donor matching (HLA-identical donor transplants showed the highest proportion of survival). While in general, studies reported that for IST and HSCT overall survival and event-free survival were similar in the two groups, HSCT in patients that received HLA-identical transplants resulted in higher survival than patients receiving IST. Adjusting for quality of life, HSCT patients enjoyed longer periods without symptoms or drug toxicity than IST patients. In the past, most patients received IST due to the inability to find an HLA-identical donor, but with scientific advancement in combatting GVHD and rejection and improved survival in transplants involving unrelated donors, HSCT is being more frequently used.

NOVEL CELLULAR THERAPIES

Clinicians and researchers are working towards developing novel therapies to cure ABMFD. The goal of novel cellular therapies is to increase patient accessibility, improve feasibility, and reduce procedure related complications. The methods range from improvements upon traditional methods, such as haploidentical transplantation, amplified umbilical blood transplantation, and mesenchymal cell transplantation, to novel ideas such as thrombocyte stimulator and chimeric antigen receptor T-cells.

HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTATION IN COMBINATION THERAPY

Haploidentical HSCT has been used frequently in the past, for patients that are unable to find a HLA identical

donor[22]. Haploidentical HSCT, by itself, leads to the great amount of complications due to unmatched HLA, such as GVHD, graft failure, or infection, resulting in significant morbidity or mortality[23]. Recently, haploidentical HSCT has been used in combination with immunosuppressive techniques to counteract the side effects of unmatched HLA. The overall goal of these combination therapies is to induce acceptance of unmatched donor stem cells in the recipient's bone marrow *via* conditioning.

Full or partial T-cell depletion in combination with non-myeloablative haploidentical HSCT has shown good preliminary results[24,25]. Immunotoxins are used to fully or partially eliminate the T-cells of the HSCT recipient before the transplant. After the transplant, immunosuppressive agents such as ATG, CSA, or various anti-complement anti-bodies or complement blockers are given on a regular basis to prevent GVHD or rejection of the stem cells. "Megadose" haploidentical HSCT along with full T-cell depletion has also been explored[24,25]. Patients showed success in stem cell engraftment, but they experienced delayed immune reconstitution and higher rate of rejection compared with using partial T-cell depletion with normal HSCT. Variations of these types of therapies are currently being explored; some of them have showed impressive result comparable with HLA matched donor stem cell transplantation. The advantages of haploidentical HSCT combination therapy are the short waiting period in finding a donor and the brevity of the entire HSCT procedure, compared with other methods.

AMPLIFIED UMBILICAL CORD BLOOD STEM CELL TRANSPLANTATION

Umbilical cord blood HSCT offers an option to patients without a HLA matched donor. The recipients of HLA unmatched umbilical cord HSCT have significantly decreased risk of GVHD or graft failure compared to matched unrelated donor HSCT. Typically, cord blood HSCT from one donor is only sufficient to treat children or small adults. Larger adults must receive amplified cord blood from two or more donors.

MESENCHYMAL STEM CELL TRANSPLANTATION

Mesenchymal stem cells (MSC) are found in the bone marrow and fat and are capable of differentiating into hematopoietic cells. MSCs represent a very small proportion of all adult bone marrow cells (< 0.1%), and their exact anatomical location within the bone marrow has yet to be determined[26]. These cells are multipotent and can differentiate into osteoblasts, fat and cartilage, in addition to hematopoietic cells. When transfused into a recipient, MSCs have a tendency to migrate to areas of injury or inflammation and proliferate into resident progenitor cells, but do not induce lymphocyte differentiation, thus immune cells such as T-cells or natural killer cells do not target MSC cells. The MSCs tendency to migrate to injured and inflammatory areas also represents a downside of using this transplantation, leading to poor engraftment. MSC can be used to enhance engraftments after HSCT. Efforts have been made to overcome these difficulties by selecting homogeneous populations of MSCs that exhibit strong osteoblastic potential, through identifying and selecting cells expression of certain surface antigens (such as STRO-1 or STRO-3)[27].

THROMBOCYTE STIMULATOR

For AA and PNH patients, drugs that stimulate thrombocyte production have been shown to have clinical benefits by improving blood clotting and raising blood cell levels for patients that have failed all standard therapies. This therapy provides a salvage option for AA or PNH patients, who are ineligible for immunosuppression and HSCT[28,29]. The drugs mimic thrombopoietin, which is the principal regulator of thrombocyte production, by binding of the receptor c-MPL on megakaryocytes. Initial clinical trials have shown a median increase in platelet count of 44000 per cubic millimeter for patients receiving the drug. Interestingly, it was observed that 8 of the 11 patients sensitive to the drug kept their response in a median of 10 mo. These drugs have been shown to stimulate erythrocyte and thrombocyte production[28] and are very helpful for patients who are unable to receive stem cell transplantation.

CONCLUSION

ABMFD is a group of rare but serious hematological diseases with a manifestation of insufficient blood cell formation. There are three main forms of ABMFD that share a similar clinical presentation and bone marrow histological appearance. The primary goals in treating ABMFD are to remove the underlining etiologic factors and to rebuild a healthy bone marrow for normal hematopoiesis. Stem cell transplantation is the ideal method to treatment ABFD. However, the high treatment related mortality, long-term complications such as GVHD, and lack of HLA matched donor sources hinder the practical use of this treatment option. With advances in cellular therapy, immunotherapy, and personalized medical therapy, novel gene modification/targeting therapy under precision medicine model opens a new frontier for ABFD therapy.

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ORIGINAL ARTICLE

Retrospective Study

Retrospective study of a cohort of adult patients with hematological malignancies in a tropical area

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Informed consent statement: We did not obtain individual informed consent; we used current hospital medical files; all the data presented were rendered anonymous and the chance of patient identification was extremely low.

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Abstract

AIM: To review the characteristics of hematological malignancies in tropical areas, and to focus on the specific difficulties regarding their management.

METHODS: This is a retrospective narrative review of cases of patients with hematological malignancies. All medical files of patients with malignant disease whose treatment was coordinated by the Hemato-Oncology service of the Cayenne Hospital in French Guiana between the 1st of January 2010 and the 31st of December 2012 were reviewed. Clinical data were extracted from the medical files and included: Demographic data, comorbidities, serological status for human immunodeficiency virus, human T-lymphotropic virus 1 (HTLV1), hepatitis B virus and hepatitis C virus

infections, cytology and pathology diagnoses, disease extension, treatment, organization of disease management, and follow-up. The subgroup of patients with hematological malignancies and virus-related malignancies were reviewed. Cases involving patients with Kaposi sarcoma, and information on solid tumor occurrence in virus-infected patients in the whole patient population were included. Since the data were rendered anonymous, no informed consent was obtained from the patients for this retrospective analysis. Data were compiled using EXCEL® software, and the data presentation is descriptive only. The references search was guided by the nature of the data and discussion.

RESULTS: In total, the clinical files of 594 patients (pts) were reviewed. Hematological malignancies were observed in 87 patients, and Kaposi sarcoma in 2 patients. In total, 70 patients had a viral infection, and 34 of these also had hematological malignancies. The hematological diagnoses were: Multiple myeloma in 27 pts, lymphoma (L) in 43 pts, myeloproliferative disorders in 17 pts and Kaposi sarcoma in two patients. The spectrum of non-Hodgkin lymphomas (NHL) was: Burkitt L (1 pt), follicular L (5 pts), chronic lymphocytic leukemia (5 pts), high-grade NHL (9 pts), mucosa-associated lymphoid tissue NHL (4 pts), T-cell lymphoma (4 pts), Adult T-cell lymphoma-leukemia (ATL)/lymphoma/leukemia (12 pts); three patients had Hodgkin disease. The spectrum of myeloproliferative diseases was: Chronic myelogenous leukemia (8 pts), thrombocytemia (5 pts) and acute leukemia (4 pts). There were no polycythemia vera, myelosclerosis, and myelodysplastic diseases. This appears to be due to bias in the recruitment process. The most important observations were: The specificity of HTLV1- related ATL malignancies, and the high incidence of virus infections in patients with hematological malignancies. Further, we noted several limitations regarding the treatment and organization of disease management. These were not related to the health care organization, but were due to a lack of board-certified hemato-oncology specialists, a lack of access to diagnostic tools (*e.g.*, cytogenetic and molecular diagnosis, imaging techniques), the unavailability of radiotherapy, and the physical distance from mainland France. Yet the geography and cultures of the country also contributed to the encountered difficulties. These same limitations are seen in tropical countries with low and intermediate household incomes, but they are amplified by economic, social, and cultural issues. Thus, there is often little access to diagnostic procedures, adequate clinical management, and an unavailability of suitable medical treatments. Programs have been developed to establish centers of excellence, training in pathology diagnosis, and to provide free access to treatment.

CONCLUSION: Management of hematological malignancies in tropical areas requires particular skills regarding specific features of these diseases and in terms of the affected populations, as well as solid public health policies.

Key words: Tropical hematology; Multiple myeloma; Non-Hodgkin lymphomas; Chronic lymphoid leukemia; Adult T-cell-lymphoma-leukemia; Hodgkin disease; Chronic myeloid leukemia; Acute leukemia; Human T-lymphotropic virus 1; Human immunodeficiency virus

Core tip: Management of hematological malignancies is guided by very specialized and up to date guidelines that are based on the biology of the diseases. An important proportion of these diseases are related to viral infections, and this is particularly so in tropical areas. Based on a narrative review of 87 cases of patients managed in French Guiana, we provide an overview of the most important characteristics of these hematological diseases (*e.g.*, human immunodeficiency virus and human T-lymphotropic virus 1 related diseases), the limitations regarding management (*e.g.*, board-certified specialists, pathology labs, imaging techniques, radiotherapy), and possible solutions to improve quality (*e.g.*, centers of excellence, training programs in pathology). These observations may be more broadly relevant in the setting of countries with low and intermediate household incomes.

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INTRODUCTION

French Guiana is an Overseas Department (ZIP code: 9700 Guiana) and a Region of France. The political, administrative, and health care organizations are the same as in mainland France. The territory covers 85000 km^2, 95% of which is Amazonian forest. The climate is equatorial. The official population is 229000 inhabitants[1], but there are approximately 40000 illegal immigrants. The population is located primarily along the Atlantic shore. There are three major cities: Cayenne and surrounding area (127000 inhabitants), Saint-Laurent du Maroni (33700 inhabitants) and Kourou (25900 inhabitants). The remainder of the population reside in small villages (2000 to 8000 inhabitants in the general vicinity of the village, and sometimes as little as a few dozen people in the village itself). The majority of the population and associated economic activities are concentrated on the Atlantic Ocean Coast (which is 450 km in length and 30 km wide). The population of French Guiana is expanding[2]. It was 115000 in 1990 and was 229000 at the last census in 2009. In 2040, the population is projected to be 480000 to 650000[3].

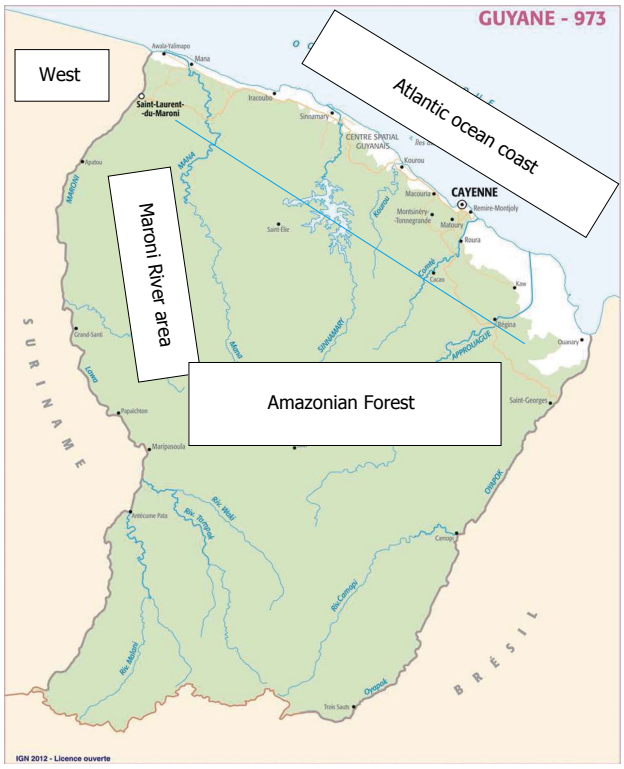

Figure 1 French Guiana map. From: Institut national de l'information géographique et forestièr. IGN: Open licence.

The median age is 28 years. The fertility rate is 3.57 children/woman. The annual population growth rate is 3.9%. The percentage of people 70 years of age or older is 1.5%. The annual birth-rate is 30.4 per 1000 and the annual death-rate is 3.7 per 1000[2]. Economic activity is based on agriculture (0.8%), panning for gold, construction (13.7%), and tertiary activities represented mainly by administrative and military entities (85.5%). The rate of unemployment is high[2]. The population of French Guiana is diverse and comprised of Guianese and French West Indies creoles (40%), metropolitans (10%), Haitians (10%), Brazilians (10%), Surinamese (10%), Chinese, Guyanese, and two specific indigenous populations groups: Native Americans (around 5000-8000 individuals) and Bushinengue ("Noirs marrons" or maroons) (around 15000 individuals). There are major cultural differences between these various ethnic groups.

There are two public hospitals (in Cayenne, and Saint-Laurent du Maroni) and one Red-Cross hospital in Kourou. The Cayenne regional hospital is a university hospital within the framework of the University of the French West Indies - Guiana. The most developed and active medical services are (1) related to obstetrics and pediatrics; and (2) emergency, intensive care unit (ICU), management of trauma and transport of wounded patients. Eighteen health care centers are linked with the Cayenne Hospital, and they are located throughout French Guiana. Figure 1 provides a map of French Guiana.

This study is part of a larger retrospective study of 594 adult patients with cancer who were managed by the Hematology-Oncology unit of the Cayenne Hospital during 2010 to 2012. The objective was to highlight the major problems regarding management of adult hematological malignancies in a tropical region with a European health organization that was subject to specific limitations due to the tropical setting and the distance from mainland France. These are: An absence of conventional and ICU hematological services, unavailability of board-certified specialists (except monthly visits by specialists from the Centre Léon-Bérard in Lyon, France), a lack of specific labs and radiotherapy on the one hand, as well as the prevalence of tropical infectious diseases and problems due to cultural diversity on the other hand. In this study we have also provided insights regarding virus related malignancies [human immunodeficiency virus (HIV), human T-lymphotropic virus 1 (HTLV1), and a focus on hepatitis B virus/hepatitis C virus (HBV/HCV) lymphoproliferative disorders]. In the Discussion section, we strived to extend the observations made in French Guiana to problems encountered in other tropical countries in the developing world.

MATERIALS AND METHODS

For the purpose of this study, we retrospectively reviewed the clinical charts of all adult patients who were managed by the Hematology-Oncology unit of the Department of Amazonian Health of Cayenne Hospital from the 1st of January 2010 until the 31st of December 2012.

The data collected were: Date of birth, age, gender, home address, place of birth, description and number of comorbidities, cancer type, extension (local, regional, metastatic or specific classification), HIV, HTLV1 and HBV/HCV serological status (no information on EBV infection status), treatment and management in mainland France or the French West Indies. Cultural identity (*e.g.,* Bushinengue or Native American) was annotated if the patient mentioned the fact during the course of the clinical management. Neither the cytology nor pathology examinations were reviewed centrally. The pathology reports were used as provided in the medical files. Examinations were performed at the Cayenne Hospital and in various laboratories in France. Details of biological characteristics of the malignancies (*e.g.,* immunochemistry and immunophenotypes, molecular biology examinations) were rarely available. Available examinations are provided in the Table 1. We restricted the analysis to descriptions; no comparison test was used. The files were rendered anonymous. Complete remission (CR) status, the date of the most recent news and follow-up (FU) duration in years, as well as the clinical status were recorded. Unfortunately, the majority of patients were lost to FU. Clinical files were summarized in an EXCEL® data base. The results were derived using features of the EXCEL® software.

We did not obtain individual informed consent; we used current hospital medical files; all the data presented were rendered anonymous and the chance of

Table 1 Hematological disease distribution and status of accessible and non-accessible examinations

Diagnosis	No. of patients	
		Examinations available in French Guiana
Myeloma	27	Complete blood count with cytology
Lymphoma (43 patients)		Bone-marrow aspiration and biopsy
Burkitt	1	Common limited immunophenotypes
Follicular	5	Standard blood chemistry
CLL	5	LDH and β2-microglobulin
High-grade B-NHL	9	Standard immunological studies
MALT NHL	4	Immunoelectrophoresis (blood and urine)
T-NHL	4	Serological testing for HIV, HTLV1, HBV, HCV, EBV, as well as PCR/RT-PCR for these viruses, HHV8, HPV and the majority of infectious diseases (opportunistic or not)
		Standard bone X-rays
		CT scan
		MRI
		Examinations not available in French Guiana
ATL/lymphoma	10	PET scan
ATL/leukemia	2	Complete immunophenotypes
Hodgkin	3	Genetic testing
Myeloproliferative diseases (17 patients)		
CML	8	
Thrombocytemia	5	
Acute leukemia	4	
Specific HIV-related entity (2 patients)		
Kaposi	2	

CLL: Chronic lymphocytic leukemia; NHL: Non-Hodgkin lymphoma; ATL: Adult T-cell lymphoma-leukemia; CML: Chronic myeloid leukemia; PET: Positrons emission tomography; MRI: Magnetic resonance imaging; MALT: Mucosa-associated lymphoid tissue; HIV: Human immunodeficiency virus; HTLV1: Human T-lymphotropic virus 1; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CT: Computed tomography; LDH: Lactico-DeHydrogenase; PCR: Polymerase chain reaction; RT-PCR: Real-time polymerase chain reaction; EBV: Epstein-barr virus; HHV8: Human herpes virus type 8; HPV: Human papilloma virus.

patient identification was extremely low.

A reference search was conducted using PUBMED with the key words: "French Guiana; cancer; neoplasm; Kaposi; lymphoma; leukemia, multiple myeloma, myeloproliferative diseases". However, we also reviewed the literature relating to recommendations published for hematological malignancies (National Cancer Centers Network - NCCN[4-7], French Hematological Recommendations[8]). Epidemiological data were derived from Globocan 2012[9,10] and from the French National Cancer Institute report of 2014[11]. The authors declare no conflict of interest.

In total, we reviewed 594 medical files of patients with malignancies, of whom 87 had hematological malignancies. We also included two cases of HIV-related Kaposi sarcoma. For the purpose of having a review of systemic virus infections in these patients, we specifically analyzed the group of patients with an HIV, HTLV1, HBV or HCV positive serological status. The entire 594 patient study was the subject of a thesis[12] for an MD degree (Bianco L), and it was reviewed and approved by the University of French Guiana and West Indies Medical School Institutional Review Board.

This article provides a narrative review of general information for the entire 89 patient cohort, and specific information on the various groups of diseases.

RESULTS

There were 50 men and 49 women; the median age was 46 years (ranging from 18-85 years of age). The majority of patients (61) lived in the Cayenne area; 15 patients lived in the Western part of French Guiana (*i.e.,* Saint-Laurent du Maroni and Mana areas) and 6 patients lived in the Maroni River area (refer to Figure 1). In 6 cases, the home address was unknown. The place of birth was the Cayenne area for 19 patients, the Western part of French Guiana for 10 patients, the Maroni River area for 6 patients, unknown for 25 patients, and countries other than French Guiana for 29 patients (Surinam 9, the French West Indies 5, Haiti 4, Brazil 3, mainland France 3, and other foreign countries for 5 patients). Comorbidities were frequent in this population of young patients: 28 had one comorbidity, 11 had 2, 8 had 3, and one had 4, while 40 had no comorbidities (for one patient, comorbidities remained unknown). The most frequent comorbidities were hypertension (25 patients), diabetes (16 patients), congestive heart failure (5 patients), lung diseases (asthma and chronic obstructive bronchopathy in 2 and 3 patients, respectively), stroke in 4 patients, and dementia and psychosis in one patient for each of these mental conditions. Two patients had chronic renal insufficiency (creatinine clearance < 30 mL/min). Tuberculosis, malaria, and leprosy were present in one patient for each of these conditions. One patient had an albinism, one had cirrhosis related to chronic HBV hepatitis, and three patients suffered of drepanocytosis.

The HIV, HTLV1, and HBV/HBC serological status were unknown in 31, 40, and 34 patients, respectively.

Table 2 Characteristics, treatment and evolution of 22 patients with active multiple

No.	Gender	Age	Ig	Stage	Regimen 1	Regimen 2	Regimen 3	FU (yr)
1	M	65	IgAλ	ⅢB	Bortezomib + DEXA	Melphalan DEXA	0	6
2	F	60	IgGκ	NA	Bortezomib + DEXA	0	0	3
3	M	66	IgAκ	NA	Bortezomib + DEXA	0	0	< 1
4	F	77	IgAκ	ⅢB	Thalidomide + Melphalan	Melphalan + DEXA	0	< 1
5	F	80	NA	ⅢA	Thalidomide	0	0	< 1
6	M	56	IgAκ	ⅢB	Thalidomide + DEXA	Thalidomide + Melphalan	0	2
7	F	71	IgGκ	NA	Bortezomib + DEXA	Thalidomide	0	4
8	F	85	IgGκ	ⅠA	Melphalan + DEXA	0	0	6
9	F	62	IgAλ	NA	Melphalan + DEXA	Bortezomib + DEXA	0	7+
10	M	83	NA	NA	NA	0	0	< 1
11	F	65	IgGλ	ⅢA	Bortezomib + DEXA Thalidomide	VAD	0	11
12	F	81	NA	ⅢB	Thalidomide + Melphalan + DEXA	0	0	< 1
13	M	57	IgGκ	NA	Bortezomib	0	0	6
14	F	69	IgGκ	ⅢA	Melphalan + Thalidomide,	Lenalidomide	0	4
15	F	59	IgGκ	ⅢA	Bortezomib + DEXA + Thalidomide	HDCT	0	3+
16	M	44	IgAκ	ⅢB	Bortezomib + DEXA	HDCT	0	3
17	F	60	IgGκ	ⅢB	DEXA+ Melphalan + Thalidomide	HDCT	Lenalidomide	6
18	M	52	IgGλ	ⅢA	Bortezomib + DEXA	HDCT	0	2
19	F	61	IgGκ	ⅢB	Bortezomib + DEXA + Thalidomide	HDCT	0	2
20	M	62	NA	NA	Bortezomib + DEXA	HDCT	Lenalidomide	2
21	M	53	IgAκ	ⅢA	VAD	HDCT	0	12
22	F	55	IgGκ	ⅢA	Bortezomib + DEXA + Thalidomide	HDCT	0	< 1

M: Male; F: Female; age in years; stage according to Durie-Salmon; Ig: Immunoglobulin; FU: Follow-up (+: died); DEXA: Dexamethasone; HDCT: High-dose chemotherapy; VAD: Vincristine, adriamycine, dexamethasone; NA: Not available.

Nine out of 58 patients had an HIV positive serological status and viremia, four of whom had been treated and had achieved infection control. Fifteen out of 49 patients had HTLV1 positive serological status. Ten out of 55 patients had a positive hepatitis virus status: 8 were HBV^+/HCV^- and two were HBV^-/HCV^+. One patient had a positive serological status for both HIV and HTLV1. One patient had previously had prostate cancer.

The hematological malignancy diagnoses are detailed in the Table 1.

Multiple myeloma

Twenty-seven patients were managed during this period: 10 men and 17 women. MGUS was not included in this series. Median age was 62 years (ranging from 44 to 85 years of age). The number of comorbidities was 0, 1, 2 and 3 in 10, 10, 4 and 3 patients, respectively. There were 10 cases of hypertensions, 5 involving diabetes, one with drepanocytosis and one with chronic renal insufficiency. One patient had a solitary plasmocytoma, IgGκ, in the sphenoid bone of the skull base, 4 patients had smoldering myeloma and 22 patients had active myeloma, 8 of whom were eligible for high-dose chemotherapy (HDCT). The monoclonal component was IgAλ in 3, IgAκ in 5, IgGλ in 2 and IgGκ in 10 patients, while this information was not available for 6 patients. The Durie-Salmon stage[13] was IA in 4 patients, ⅢA in 7, ⅢB in 7 and not available for 8 patients. One patient was HIV^+, but had achieved disease control following treatment, while one patient had an HBV^+ serological status. The patient with a plasmocytoma had a partial resection of the tumor and radiotherapy followed by adjuvant Bortezomib treatment for 6 mo. Patients with smoldering myeloma were followed without being given a specific treatment, and one of them died two years later. Treatment and evolution of the 22 patients with active myeloma are shown in the Table 2. All these patients received biphosphonates treatment and supportive care.

Eleven patients were treated in mainland France. These were the eight patients who had high-dose chemotherapy (one cycle of high-dose Melphalan) and autologous bone-marrow support (ABMS), the patient with plasmocytoma, and two other patients.

Lymphoid malignancies

Forty-three patients had lymphomas, including adult T-cell lymphoma-leukemia (ATL)/lymphoma/leukemia and Hodgkin disease. There were 28 men and 15 women; median age was 50 years (ranging from 18 to 84 years of age).

Clinical entities are summarized in the Table 2.

Chronic lymphocytic leukemia

Five patients had chronic lymphocytic leukemia (CLL). They were four men and one woman, aged 46, 50, 60, 62 and 70 years. The Binet stage[14] was A, B, C in 2, 1 and 2 patients, respectively. One patient had auto-immune hemolytic anemia (a Bushinengue patient with stage C disease). Patients had standard immuno-phenotyping (Matutes score)[15], but none of these patients underwent fluorescence *in situ* hybridization (FISH) and molecular analysis. One patient had an HTLV1 positive serological status. Two patients were only monitored (less than 1 and 6 years FU, respectively), one received Chlorambucil (less than 1 year FU). Two patients received Fludarabine and Rituximab (3 years FU), one of whom eventually received R-CHOP (5 years FU).

Follicular lymphoma

Five patients had follicular lymphoma. They were 2 men and 3 women; median age 55 years (ranging from 40 to 66 years of age). Two patients had HBV positive serological status, one of whom had post hepatitis cirrhosis. One patient was HIV^+, although he had received treatment and achieved disease control. The Ann Arbor stage[16] was: ⅡAE (1 patient, E: Breast), ⅢA (2 patients), Ⅳ (2 patients). All of these patients were treated in mainland France. Four patients received R-CHOP and one Rituximab only.

Mucosa-associated lymphoid tissue lymphoma

Four patients (3 men and one woman; aged 42, 68, 69, 84 years) had mucosa-associated lymphoid tissue (MALT) lymphomas. Two patients had gastric MALT, Ann Arbor stages ⅡE and Ⅳ (one patient had a positive HCV and HTLV1 serological status) and two patients had skin MALT, both stage Ⅳ (one of whom had a positive serological HTLV1 status). All patients received Rituximab, on its own for one patient, and combined with Fludarabine for two patients, with CHOP either as first line or second line, depending on the patient. Three patients reached a CR status and one attained stable disease. One patient died after 2 years of disease progression, another patient died after 10 years of cardiac heart failure, and two patients underwent 4 and 9 years FU.

Burkitt lymphoma

A 46-year-old male immigrant from Senegal had a high-risk Burkitt lymphoma. He had a prior record of malaria and a controlled HIV infection. He was transferred to Paris for treatment where he received R-COPADEM-CYVE regimen chemotherapy[17]. This individual went into complete remission (at 1 year of FU).

High-grade B-cell lymphomas

High-grade B-cell lymphomas were observed in 9 patients. For 3 patients the data was incomplete, as full pathology and immunophenotyping were not available[18,19].

DLBCL was observed in 9 patients. Five patients were treated in the West French Indies or mainland France. Two patients were of Bushinengue descent, and one was a Native American. Disease extension was not available in 3 patients. The Ann Arbor stage was ⅡA and ⅢB in one patient each, Ⅳ in 4 patients. Treatment modalities were available for 7 patients: 6 patients received R-CHOP[20] and one R-ACVBP[21]. Two patients had HDCT and ABMS (2 CR) and two patients required DHAP[22] salvage chemotherapy regimen (one CR). In total there were 4 cases of CR (5, 10, 10, and less than 1 year FU), 4 other patients had less than 1 year FU, and one patient died after 2 years. The case of this latter patient is notable. He was a 53-year-old male of Bushinengue descent who lived along the Maroni River and who had an albinism. He had multiple exereses of basal-cell carcinoma of the skin. Serological status for HIV, HTLV1, HBV and HCV were all negative. He had a stage ⅢB DLBCL and received R-CHOP that was complicated by neutropenic fever. He received R-DHAP after disease progression, and died of disease progression after 2 years of this treatment.

Hodgkin disease

Two women and one man, aged 18, 44 and 51 years, had Hodgkin disease. The histological type was nodular sclerosis in all 3 patients. The Ann Arbor stages were ⅡA (2 patients) and ⅢA. The 51-year-old male had been treated for HIV infection, which was under control, and a renal insufficiency. All three patients received ABVD regimen[23] of chemotherapy. The patient with stage ⅡA disease was referred to mainland France for radiotherapy. All of them entered into CR status, but were then soon lost to FU.

T-cell lymphoma

Four patients has T-cell lymphomas, all of them had very uncommon clinical history with unfortunately a lot of missing information.

Two men, aged 40 and 75 years, had T-cell lymphoma. Both received CHOP regimen chemotherapy. The 75 year old patient had a response to treatment, but he was lost to FU after 5 years. Unfortunately, disease extension was not available. The other patient was a 40-year-old of Bushinengue descent and residing in the Maroni River area. He had an active untreated and uncontrolled HIV infection. HTLV1 and HBV/HCV serological status were negative. The Ann Arbor stage was Ⅳ with osteolytic bone involvement and hypercalcemia. The patient received CHOP chemotherapy regimen. He died, however, of progressive disease after 4 mo.

One patient had a very complex disease, for which many of the data were not available. This individual (a 49-year-old male) had respiratory insufficiency and hyper eosinophilia. Serological status was negative, and he had no parasitic disease. The lung biopsy demonstrated a bronchocentric granulomatosis. The blood cytology and in the medullary aspirate showed a monoclonal T-cell proliferation. Nonetheless the precise phenotype is not available. Various treatment sequences were administered. He was lost to FU after 10 years.

The last patient was a 21-year-old male from the Maroni River area and he was of Bushinengue descent, with an Ann Arbor stage Ⅳ lymphoma which was classified as precursor B-cell lymphoblastic lymphoma. He received R-CHOP and entered into CR, and relapsed and died one year later. Nevertheless the retrospective study of the pathological report (liver biopsy) shows a profile of large-cell anaplastic T-cell lymphoma (ALK-).

ATL/lymphoma/leukemia

Nine patients had ATL/lymphoma and two patients ATL/leukemia. There were 6 women and 5 men; their median age was 46 years. Nine patients were of Bushinengue descent, eight of whom lived in the

Table 3 Characteristics, treatment and evolution of adult T-cell lymphoma-leukemia/lymphoma/leukemia

No.	Gender	Age	Stage	Tumor sites	Regimen 1	Regimen 2	Regimen 3	FU
ATL/lymphoma								
1	M	28	NA		L/Z + P-IFN + oral ETO	CHOP	0	2
2	M	34	Ⅳ		L/Z + P-IFN	CHOP	DHAP	3
3	M	38	NA		L/Z + P-IFN	CHOP	DHAP	1
4	F	46	Ⅲ	Abdomen, Ca^{2+}		CHOP	VBl	1
5	M	49	Ⅳ			CHOP	DHAP	< 1
6	M	49	Ⅳ	Colon, CNS	L/Z + P-IFN	CHOP	IT-MTX	< 1
7	F	51	Ⅰ E	Nasal, sinus	0	CHOP	0	< 1
8	F	58	Ⅱ A		L/Z + P-IFN	CHOP	0	3
9	F	62	NA		L/Z + P-IFN + ETO oral	CHOP	0	5
10	M	42	Ⅳ	Ca^{2+}	R-CHOP	0	0	3
ATL/leukemia								
10	M	41	-	Liver, cavum	L/Z + P-IFN	CHOP	0	< 1
11	F	47	-		L/Z + P-IFN + oral ETO	CHOP	0	4

Hydroxydaunorubicine, oncovin®, prednisone; M: Male; F: Female; age in years; stage according to Ann Arbor staging system; ATL: Adult T-cell lymphoma-leukemia; FU: Follow-up in years; Ca^{2+}: Hypercalcemia; CNS: Central nervous system; L/Z: Lamivudin + Zidovudin; P-IFN: Peg-interferon α-2a; ETO: Etoposide; CHOP: Cyclophosphamide, R-CHOP: Rituximab-cyclophosphamide; DHAP: Dexamethasone, aracytine, cisplatin; VBl: Vinblastine; IT-MTX: Intra-thecal methotrexate; NA: Not available.

Western part or in the Maroni River area. Four patients had hypertension; two had diabetes, one suffered from drepanocytosis. One patient had a serologically positive $HBV^{+}HCV^{-}$ infection. One patient had a treated and controlled HIV infection. All patients had serologically positive HTLV1 infection. It is noteworthy that four patients had aggressive Strongyloides stercoralis GI infections. Three patients were treated in Paris and one in the Netherlands. The patient treatments and evolution are shown in the Table 3. To date, no patient has died, while two patients are in CR.

One patient had a singular history. He was a 42-year-old man from the Maroni River area and of Bushinengue descent with a stage Ⅳ lymphoma (lymph nodes and bone osteolytic lesions). He had hypercalcemia. This patient had serologically positive untreated HIV and HTLV1 infections. He had also a retinitis and CNS toxoplasmosis. The disease was initially diagnosed as diffuse large B-cell lymphoma (DLBCL). The patient received two cycles of R-CHOP, but contact with this patient was lost soon thereafter, thereby precluding FU. But the diagnosis was reviewed and changed to an ATL/lymphoma.

Myeloproliferative syndromes

Chronic myelogenous leukemia: Eight patients had chronic myelogenous leukemia (CML). They were 5 men and 3 women, with a median age of 51 years (ranging from 37 to 82 years of age). Aside from the older patients, none of them had significant comorbidities. The diagnosis was established by cytology, and the presence of Ph1 by FISH. Three patients underwent bcr-abl transcript analysis, one of whom lacked Ph1 [he was bcr-abl(-), but JAK^{2+}], and two after developing resistance to Imatinib. The possibility of bcr-abl point mutations was, however, not addressed. The older patient, who was bcr-abl(-), received hydroxyurea. Three patients received first line hydroxyurea, followed by Imatinib and then Dasatinib. Three patients received first-line Imatinib, and then Dasatinib, while one patient received Dasatinib first-line. None of the patients were transferred to mainland France, and none died of the disease after a median FU of 3 years. None of them was considered for allogenic bone marrow transplantation.

Essential thrombocytemia: Three women and two men, aged 34, 41, 44, 49, 54 years, had essential thrombocytemia. One patient had hypertension and another had hypertension, arteritis and stroke, while the younger patient had a portal thrombosis. Three patients had a $JAK6^{V617F}$ mutation. This mutation did not occur in two patients, one of whom was bcr-abl(-). These two patients had no evaluation of MPL mutation. One patient received Anagrelide, and all of these patients were treated with hydroxyurea. They were alive after 3-4 years of FU.

Acute leukemia: Three men, one woman, aged 22, 39, 46, 54 years were diagnosed with acute myeloid leukemia. All of them were transferred to Paris for treatment. According to the FAB classification[24] there were one LAM5 and three LAM3: One of them was promyelocytic $RAR\alpha^{+}$, another had a Flt3 duplication with t(15;17). Patients were treated according to standard regimens[8]: The patient with LAM5 had an allogenic bone-marrow transplantation; patients with LAM3 received Idarubicin, all-trans-retinoic acid, and arsenic trioxide. Patients entered into CR, and they were free of disease at less than 1, 1, 2 and 5 years of FU.

Malignancies related to viral infections

The distribution of tumor types in patients infected with viruses is shown in the Table 4.

HIV infection: Nine patients had an HIV infection in this series, 4 with treated and controlled disease. Five

Table 4 Distribution of tumor types in patients infected by viruses

Virus	Hematological malignancies	Solid tumors	Total
HIV	9	19	28
HTLV1	15	4	19
HBV	8	10	18
HCV	2	3	5
Total	34	36	70

HIV: Human immunodeficiency virus; HTLV1: Human T cell leukemia/lymphoma virus 1; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

patients had lymphomas (one follicular lymphoma, two DLBCL, one ATL/lymphoma, one Burkitt lymphoma). The other patients either had myeloma or Hodgkin disease.

Two patients had the HIV-related malignancy Kaposi sarcoma. They were two men who were aged 38 and 54 years. One had an uncontrolled HIV infection. He had serous involvement by the disease, and although he received liposomal Doxorubicin, the disease progressed and this patient was soon lost to FU. The other patient, an immigrant from Haiti, had a treated and controlled HIV infection, and he had involvement of the colon, lymph nodes and particularly the skin of both legs. He received liposomal Doxorubicin and experienced stable disease. He was alive with the disease after 3 years of FU.

Of the total of 28 patients who were HIV^+ in the series of 594 patients, 9 were described above. The tumor types in the 19 other patients were: Prostate, gastric (3 each), cervix, breast, lung, head and neck (2 each), penile, kidney, pancreas, esophageal and colon cancers (one each).

HTLV1 infection: Nineteen of the 594 patients had a positive serological status. Fifteen patients had hematological malignancies, of which 12 were ATL/lymphoma/leukemia; two MALT non-Hodgkin lymphoma (NHL), one CLL. The four other patients either had colon, liver, prostate or kidney cancer.

HBV infection: Of the entire 594 patient cohort, 18 patients had a positive serological status: 8 had hematological malignancies while the others had various tumor types comprised of breast (3), liver (2), gastric (2), cervix, prostate and head and neck cancer (one each).

HCV infection: Of the entire 594 patient cohort, 5 patients had a positive serological status: 2 had hematological malignancies and the others had either liver (2), or lung cancer (1).

DISCUSSION

This study reports on 87 patients with hematological malignancies (and two with Kaposi sarcomas) who were managed during a three-year period at the main hospital in French Guiana. However the study scale is small, but it provides several interesting insights that are of considerable relevance in terms of the provision of medical care. First of all, there is the effect of bias. There are biases of recruitment of patients in the series: Patients may have been managed in the two other hospitals of the territory. This may not have occurred so much in Kourou (which is only 50 km from Cayenne), but rather in Saint-Laurent du Maroni, which is 250 km far from Cayenne. It is noteworthy that Saint-Laurent is the most substantial city (around 50-70000 inhabitants) of the Western part of French Guiana, of the Bushinengue area and also of both banks of the Maroni River (French and Surinamese), as Paramaribo, Surinam's capital is 150 km west of Saint-Laurent. Moreover, there are many illegal immigrants, but since the border control is 100 km down the road running from Saint-Laurent to Cayenne, many patients are managed at the local hospital. Other biases are the fact that myelodysplastic syndromes are either managed by other hospital wards, or they are misdiagnosed; an unknown proportion of patients are directly referred to mainland France; and lastly, an unknown proportion of patients from the Western and Maroni River areas are certainly not diagnosed due to the absence of robust medical network.

Other interesting insights are the gap between the needs for hematological disease management and the facilities that are available. This aspect can be equated with countries in tropical areas that have low and intermediate household incomes. Lastly, this series shows the importance of infections in the occurrence of some hematological malignancies to be reviewed.

Distribution of diseases

In this series, out of 594 patients with malignancy, hematological malignancies occurred in 87 patients (*i.e.,* 14.5%).

The incidence of hematological malignancies could, however, not be measured. We are cognizant of the fact that it is scientifically inexact as it is an approximate assessment of the prevalence of hematological diseases which were accounted for in the period from 2010 to 2012. In 2008, in mainland France, the incidence of hematological malignancies relative to solid tumors was found to be 6.5%[25]. The comparative morbidity figures for solid tumors in mainland France (2005) *vs* French Guiana (2003-2009) was determined to be 1.30 and 1.31 for men and women, respectively[26]. The incidence of cancers therefore seems lower in French Guiana than in mainland France. GLOBOCAN 2012 incidence data[9,10] show that French Guiana has a global incidence of cancer in keeping with other northern and North-Western parts of South America (137.5/100000), but lower than in Brazil, Argentina, Chile and specially Uruguay (where the incidence is the highest, and at a level that is similar to Western countries). The incidence of hematological malignancies in French Guiana is similar to that of

mainland France, but higher than in the majority of South American countries, at 6.5/100000 for leukemia (as in Ecuador), 2.4/100000 for multiple myeloma (as in Surinam) and 7.0/100000 for NHL (as in Colombia and Uruguay).

Multiple myeloma

Patients with multiple myeloma had the same median age as in mainland France, while there was a slight predominance among women. The precise staging procedures at the initial diagnosis were not always fully available in the medical files. Nevertheless, with the exception of the cytogenetics of plasma cells[5], the most important criteria were available. The most important genetic abnormalities should be in regard to chromosomal 13q deletion, and detection by FISH of t(4;14), t(14;16), and del17p. These are poor prognostic factors. The international staging system is nonetheless used in clinical practice[27]. The majority of patients benefited from proteasome inhibitors, and Thalidomide. Eight out of 22 patients had high-dose Melphalan with ABMS. The conditions to indicate HDCT and ABMS are based on response to first-line treatment[28] and practical opportunity to perform HDCT. Treating multiple myeloma is hence not a problem in the setting of this organization. In patients not eligible to HDCT, new regimens including targeted drugs are more active than Melphalan plus prednisone regimen[29], and a Bortezomib-DEXA regimen seems to have the same activity as the three drug regimens (with Thalidomide or Melphalan)[30].

Lymphomas

The pathology diagnosis of lymphoma was established by different pathologists in French Guiana, the French West Indies or mainland France. The standard procedure first comprised morphological analysis, followed by a standard immunophenotyping panel with at least CD20, CD79a for B-cell malignancies and the same negative readout with CD3, CD5 for T-cell malignancies. Depending on the first screening and the capabilities of the laboratory, other antigens were also tested. Precise information regarding the pathology procedures and classification was not generally available. Since 2010, the biopsy samples have been referred to one of the pathology reference centers affiliated with the French National Cancer Institute (LYMPHOPATH network). Clinical staging of lymphomas (both NHL and Hodgkin Disease) used the Ann Arbor classification, as originally reported[16], because the lack of access to TEP-scanning did not allow use of the Lugano staging system[31].

Patients with CLL exhibited classical features of the disease. There were diagnosed using an immunophenotyping panel that allowed application of the Matutes score[15].

Staging of the patients was established according to the Binet classification[15]. None of the patients had FISH to screen for a del(11q) or del(17p), which plays an important role in the identification of specific treatments, nor Ig mutational profile and expression of protein ZAP70, which are highly relevant for the prognosis[8]. Patients therefore received treatment according to their clinical status. Thus, the patient with auto-immune hemolytic anemia received R-CHOP regimen. The treatment eventually conformed to recognized standards.

Patients with follicular lymphomas received a standard work-up, including a bone-marrow aspirate and biopsy. They were staged according to the Ann Arbor classification. It is noteworthy that these patients were all referred to mainland France for treatment. There is no specific explanation for this, except that two of them were from a metropolitan area. All of them nonetheless benefited from extensive work-up, and they received Rituximab with or without a CHOP regimen of chemotherapy for extensive or progressive disease. This is standard disease management. Two patients had an HBV infection, which has not been reported to be linked with the occurrence of follicular lymphoma[32]. HIV infection also does not seem to be related to this NHL type.

Two patients had gastric MALT lymphoma. Information regarding infection by *Helicobacter pylori* was not available, and FISH t(11;18) was not performed. One of these patients had positive HCV and HTLV1 serological status (stage ⅡE). Unfortunately, no information on splenic involvement was available, as it has been described in splenic marginal zone lymphoma related to HCV infection[33]. Two patients had skin MALT lymphoma. Information on infection by Borrelia burgdorferi was not available. One of these patients had a positive serological HTLV1 status. The staging procedure was standard. With gastric MALT lymphoma, no information on *Helicobacter pylori* treatment was known, but all of them received induction immunotherapy (Rituximab). In three cases, associated chemotherapy was given because of stage IV disease.

The patient with Burkitt lymphoma had standard current characteristics: Malaria[34], African descent, and HIV infection[35-37]. He was treated in keeping with the best treatment standards in Paris[17].

High-grade lymphomas were diagnosed according to morphological aspects and standard minimal immunophenotyping. The Ann Arbor stage was used, but the International Index was not available[38]. Five patients were transferred to mainland France and therefore had a complete diagnostic and clinical work-up. When the information was available, these patients nonetheless received standard chemotherapy regimens[39] with Rituximab. Two patients with stage Ⅳ disease, who responded to conventional chemotherapy, received HDCT and ABMS. The two Bushinengue patients with specific disease were not transferred to mainland France. This may have been due to the absence of coverage for their health expenses. The native American patient was transferred to Paris for treatment. These individuals are usually French citizens, and they hence have the appropriate level of social security coverage. DLBCL belongs to the spectrum of hematological malignancies related to HIV[40], but that are unrelated to HTLV1 and HBV. The precise diagnosis of albinism was not done,

but it is likely to have been a type 2 oculocutaneous albinism. This disease does not seem to increase the risk of lymphoma (although this patient did experience repeated basal cell carcinomas).

Two patients had large cell T-cells lymphoma. Unfortunately, information regarding the expression of ALK was not available[6]. The response to CHOP was in keeping with the diagnosis of anaplastic large cell lymphoma. It is very rare to come across a T-cell lymphoma in a patient with an untreated uncontrolled HIV infection. A misdiagnosis must hence be suspected.

The three patients with Hodgkin disease did not have exceptional characteristics, and they were treated in keeping with the standard treatment[4]. One patient had a controlled HIV infection, and this might have been involved in the mechanism of the disease, although it unexpectedly exhibited a nodular sclerosis pattern, which is less frequent than mixed cellularity in this setting[41].

Eleven patients had ATL/lymphoma/leukemia, as defined by the Shimoyama classification[42]. The serological status was established by the ELISA method and confirmed by Western blot. When results from these assays were inconclusive, HTLV1 PCR was performed (at the Pasteur Institute). All patients had been infected by HTLV1. It is noteworthy that 9 of them were Bushinengue. This is a very well-recognized observation in French Guiana[43]. The Bushinengue population has a high prevalence of this infection[44], which is seen extensively in children[45] as the virus is transmitted from the mother by breast feeding[46,47]. Clinical work-up was in agreement with recommendations[6]. Four patients had Strongyloides GI involvement, which is often observed with this disease[48]. Patients received Ivermectin or Abendazole treatments. Patients also received standard treatment focused on retrovirus control, as a combination of Lamivudin + Zidovudin and Peg-interferon α-2a[49]. It also included standard chemotherapy regimens, such as CHOP[50] as first-line and DHAP as second-line treatments[6]. Five patients were treated in mainland France. This may present a difficult socio-cultural problem for the patients who live in the Maroni River area, and is an issue which will be discussed below. Due to cultural reasons, the majority of patients were lost to FU.

Myeloproliferative syndromes

The eight patients with CML were managed in a very conventional manner. Unfortunately, they were underdiagnosed and potentially undertreated. Apart from the cytological diagnosis and Ph1 FISH, the initial work-up ought to have included analysis of bcr-abl transcripts and screening for JAK2, CALR and MPL mutations, as well as a complete karyotyping[7]. No patient underwent mutational analysis for TKI second-line resistance so as to evaluate further indication of Nilotinib, Bosutinib, Ponatinib and Omacetaxine. Criteria for cytogenetic[51] and molecular[52] responses were not performed. Therefore, patients received Hydroxyurea and/or first-line Imatinib, and Dasatinib when they progressed. The profile of the patients did not fit with conditions for treatment by allogenic bone-marrow transplantation. Furthermore, organizing a prolonged stay in mainland France to select the indication and to perform the procedure is fraught with difficulties[53].

Surprisingly, we observed five cases of thrombocytemia. The same criticism can be made in terms of the lack of a complete work-up to at least eliminate secondary thrombocytopenia[8]. These diseases are however not specific to this tropical region.

The failure to encounter cases of polycythemia vera, myelofibrosis and myelodysplastic syndromes is certainly due to a bias in the recruitment process; such as the patients not being referred to the unit, not been diagnosed or, in some cases being directly referred to mainland France by their treating physician.

Lastly, four patients had acute myeloid leukemia. All of them were transferred within two or three days after diagnosis to a hematology unit in Paris by medical transport using the daily flight between Cayenne and Paris. This may present a substantial bias since these four patients were adults who had achieved CR status, while some patients may have been transferred only to return directly home with progressive disease, or they may even have died while in Paris. Further, some patients may have been children who were not managed in the hematology-oncology unit, while some patients may have died prior to diagnosis due to the distance from their residence to the hospital (for example, a patient who lives in the Maroni River area, with no doctor nearby, could face a two days trip by canoe to Saint-Laurent du Maroni, and then at least another day for administrative processing and a day for transfer to Cayenne…).

Viruses and hematological malignancies

The importance of viral infections in this patient population from this tropical region has been reported in various epidemiological studies[32]. Half of the virally-infected patients presented with hematological malignancies, while only 14% of the entire patient population of the series exhibited hematological malignancies. HIV is the most common viral infection. Hematological B-cell malignancies correspond with being afflicted with AIDS[40]. Two patients had very aggressive Kaposi sarcomas, which are typically encountered with HIV infected patients[54]. In this series, HBV serum positive status is often encountered, but there is no evidence in the literature that this virus could be involved in the occurrence of hematological malignancies. HCV has however been implicated in the occurrence of marginal B-cell lymphomas[33]. The small number of positive patients does, however, not allow such a conclusion to be drawn from this series.

Conversely HTLV1 infection is quite common. Moreover, screening for HTLV1 infection is part of the diagnosis of ATL/lymphoma/leukemia. Although serological positivity in French Guiana is quite high, this does not necessarily mean that affected individuals are

going to develop hematological malignancies. Indeed, the majority of patients are highly unlikely to suffer any adverse consequences from this infection.

Practical management

The Table 1 aims to give an indication of what is available for the diagnosis of patients with hematological malignancies. Thus, basic examinations are reasonably available, while some of the more in depth examinations may be obtained at a metropolitan laboratory. What is most relevant, however, is to have one or two board-certified (hemato-oncology) specialists available to manage the patient, specify the work-up, monitor patient progression, and get feedback from colleagues in mainland France regarding the patient cases. Lastly, they can recommend the transfer of patients for specialized management. This is not currently the case. At present, this is done by monthly 3 d visits of specialists from Centre Léon-Bérard in Lyon, and weekly contact with the tumor board by video conferencing. The current daily management is performed by two generalist practitioners. The issues encountered require a medico-scientific view of the situation. A very strong relationship between the Hemato-Oncology unit in Cayenne and a center of excellence in mainland France is paramount in providing these patients access to optimal treatment. Two useful logistical entities can be developed on site: Extension of the laboratory of pathology, and the implementation of TEP-scanning. Conversely, implementation of radiotherapy is not feasible to date.

Thus, in this regard French Guiana is, in fact, fully linked with mainland France, and can be seen as being independent of its neighboring countries Surinam and Brazil. Indeed, there are no transportation means to readily reach urban centers in Brazil. Although there actually is a bridge across the Oyapock River (at a distance of 200 km from Cayenne), there is no paved road to Belem (600 km). Moreover, the health care system in Brazil is mainly private. Only illegal Brazilian immigrants could benefit from having their disease managed in Brazil. Cooperation with Surinam could be easier to organize, as a Surinamese company operates flights between Cayenne and Paramaribo. It is also feasible to cover this 400 km distance by motor vehicle. Such an arrangement would however also require a genuine willingness to cooperate at a political level, and this has not been scheduled to date.

Socio-cultural problems

Social aspects are very important considerations. The majority of patients, even illegal immigrants, are covered by one or other of the various national health insurance systems. In French Guiana 60%, 28%, 12% of the population is covered by the national health insurance, the universal social insurance and the emergency medical help, respectively. This means that the administrative management for these patients is extremely time consuming and needs multiple players, including the police, social security, social services, *etc.*

Cultural aspects are also very important. The recommendations produced by the French National Cancer Institute for cancer patient management have been implemented, and they match international criteria. This nonetheless only applies to a small proportion of patients, being mainly the residents of metropolitan areas and a small proportion of Guianese and French Caribbean creoles. Even in this population the word "cancer" is taboo. This applies even more to Bushinengue and native Americans patients. In general, knowledge of the illness is expressed clearly (using the word "cancer"). The problem lies more with the significance of such a diagnosis, particularly when it comes to interpretation of the cause of the disease, which is often assigned to evil spirits or somebody who has cast a spell[55]. A great proportion of patients therefore seek advice from a wizard or medicine man. This does not preclude the possibility of conventional treatment, although it does generally delay it. These aspects are inadequately scrutinized and overcome by cancer patients. More progress has been made for patients with HIV infections[56]. An important aspect is that it is often thought that Bushinengue patients who become ill should return to their own village to die. This view hence tends to fully preclude taking the risk that the patient might die elsewhere, such as in a hospital, possible far away, such as in mainland France. The view is often that, should this occur, it would cast a pronounced spell on the deceased's lineage. It is therefore important that transcultural mediation is developed, as has been done for HIV patients[56].

Translation to management of patients with hematological malignancies in tropical countries with low and intermediate household incomes

This series summarizes some of the problems that are encountered in other tropical countries that have low and intermediate household[57]. Unlike French Guiana, these countries do not have the option, however, of providing disease management in mainland France. In these countries the most severe shortcomings are the lack of a sufficient number of board-certified specialists, the equipment and skill of pathology laboratories, the lack of access to new drugs, and particularly targeted drugs (mainly due to cost), as well as the lack of imaging and radiotherapy equipment. Various initiatives have been instigated to implement centers of excellence[58] and programs to provide access to new drugs, as done for Imatinib in CML and GIST[59,60]. The implementation of radiotherapy is also an important initiative, as it makes one of the most efficient therapeutic means more accessible[61]. Another important initiative is the implementation of diagnostic pathology learning programs. These are promoted by the International Network Cancer treatment and Research[62]. Initiative have been promoted for surgery training[63]. Training and research programs are priorities of international organizations[64,65].

In conclusion the specificity of tropical hematology (and oncology) is important: Firstly, in terms of gaining knowledge and understanding of disease mechanisms,

and, secondly for decision making and organization of the management of these malignancies in the tropical areas. The latter requires particular skills relating to the specificity of these diseases and of the affected populations, as well as solid public health policies. Finally the study reflects the problems the hematologists face in the daily practice in this area.

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COMMENTS

Background

This study is part of a larger retrospective study of 594 adult patients with cancer who were managed by the Hematology-Oncology unit of the Cayenne Hospital during 2010 to 2012.

Research frontiers

The objective was to highlight the major problems regarding management of adult hematological malignancies in a tropical region with a European health organization that was subject to specific limitations due to the tropical setting and the distance from mainland France.

Innovations and breakthroughs

This aspect can be equated with countries in tropical areas that have low and intermediate household incomes. Lastly, this series shows the importance of infections in the occurrence of some hematological malignancies to be reviewed.

Applications

In conclusion the specificity of tropical hematology (and oncology) is important: Firstly, in terms of gaining knowledge and understanding of disease mechanisms, and, secondly for decision making and organization of the management of these malignancies in the tropical areas. The latter requires particular skills relating to the specificity of these diseases and of the affected populations, as well as solid public health policies.

Peer-review

It is an interesting retrospective study regarding hematological malignancies in french guiana. The study also reflects the problems the hematologists face in the daily practice.

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MINIREVIEWS

Role of microRNA in regulation of myeloma-related angiogenesis and survival

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Abstract

Multiple myeloma (MM) is a malignant disease caused by clonal proliferation of plasma cells that result in monoclonal gammopathy and severe end organ damage. Despite the uniform clinical signs, the disease is very diverse in terms of the nature and sequence of the underlying molecular events. Multiple cellular processes are involved in helping the malignant cells to remain viable and maintain proliferative properties in the hypoxic microenvironment of the bone marrow. Specifically, the process of angiogenesis, triggered by the interactions between the malignant MM cells and the stroma cells around them, was found to be critical for MM progression. In this review we highlight the current understanding about the epigenetic regulation of the proliferation and apoptosis of MM cells and its dependency on angiogenesis in the bone marrow that is carried out by different microRNAs.

Key words: Multiple myeloma; MicroRNA; Angiogenesis; Proliferation; Apoptosis; Hypoxia; Vascular endothelial growth factor; Hypoxia-induce factor 1α; Macrophages; Endothelial cells

Core tip: The pathogenesis of multiple myeloma (MM) requires that malignant cells remain viable and proliferate. Therefore, genes relating to the regulation of apoptosis, proliferation and angiogenesis are tightly regulated. Specifically, angiogenesis, which is driven by the interactions between the malignant cells and stroma cell surrounding them, is critical for MM progression. In this review we summarize the current knowledge about the regulation of the expression of genes related to apoptosis, proliferation and angiogenesis, through the activity of specific microRNAs.

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INTRODUCTION

Multiple myeloma (MM) is an incurable B-cell neoplasm, where clonal plasma cells proliferate mostly within the bone marrow and produce high amounts of monoclonal paraprotein. Symptomatic myeloma is characterized by the presence of more than 10% clonal plasma cells on a bone marrow biopsy and the presence of end organ damage spanning hypercalcemia, renal insufficiency, anemia and bone lesions. Recently the International Myeloma Working Group added additional criteria to define the symptomatic disease[1]. The disease itself always starts as a premalignant condition termed Monoclonal Gammopathy of Undetermined Significance (MGUS), characterized by a low number of bone marrow plasma cells, with low levels of monoclonal protein production. As the disease progresses the number of clonal plasma cells in the bone marrow increases. When the clonal plasma cells are more than 10% of the cells in the marrow with no evidence of the symptomatic disease it is termed "Smoldering Myeloma"[2].

Despite the homogeneity in MM appearance, symptoms and disease progression, from a molecular standpoint, MM is a group of molecularly distinct diseases, with similar phenotypic characteristics. The events that generate the terminal state of MM are heterogeneous and diverse, and consist of hyperdiploidy, chromosomal aberrations such as translocations (especially those involving the immunoglobulin heavy chain locus at 14q32), chromosome deletions (such as in chromosome 13 or chromosome 17p), or combinations of translocation and dysregulation of at least one of the cyclin D genes. The latter is used to classify MM patients according to their translocation/cyclin (TC) status[3]. These changes have significant prognostic implications, as patients with high risk disease changes such as translocation t4;14 and deletion of 17p have dismal prognosis[4]. This diversity, of course, renders the study of the pathophysiology of the disease more difficult.

IMPORTANCE OF ANGIOGENESIS IN THE PATHOGENESIS OF MM

Angiogenesis, as a means of supplying oxygen and nutrients to the growing number of tumor cells, exists not only in solid tumors, but also in hematological malignancies, such as MM. Indeed, increased microvessel densities and elevated levels of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), insulin-like growth factors-1 (IGF-1), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony stimulating factor in the bone marrow and serum of MM patients have been reported, and were associated with poor prognosis[5-7]. Specifically, VEGF is overexpressed in malignant plasma cells, and acts in a paracrine manner to enhance the proliferation and chemotaxis of endothelial cells, as well as other stroma cells[5].

Existing therapies have anti-angiogenic properties

Existing therapeutic approaches to this disease include immunomodulatory drugs such as thalidomide, lenalidomide and pomalidomide, as well as proteosome inhibitors such as Bortezomib. These drugs target pro-angiogenic factors and have known anti-angiogenic properties, suggesting that their efficacy is at least partly due to their ability to block angiogenesis in MM development. Early experiments demonstrated the anti-angiogenic potential of Thalidomide[8]. Lenalidomide was also found to inhibit migration and invasion of endothelial cells in a dose-dependent manner, as well as inhibiting VEGF-induced PI3K-Akt pathway signaling[9]. Pomalidomide inhibits stromal cell adhesion and has been shown to markedly inhibit angiogenesis by decreasing concentration of VEGF and hypoxia-induce factor 1α (HIF-1α)[10]. At pharmacological doses, bortezomib was found to inhibit endothelial cells proliferation, migration, and capillary formation. Bortezomib was also shown to decrease secretion of VEGF and IL-6 from myeloma cells[11].

Angiogenesis is driven by interactions between tumor and stroma cells

The bone marrow microenvironment is heterogeneous, and consists of different immune cells (NK cells, B and T lymphocytes, monocytes, and dendritic cells), erythrocytes, hematopoietic stem cells, bone marrow mesenchymal stem cells, endothelial cells (ECs) and their precursors, fibroblasts, osteoblasts and osteoclasts - all closely associated with the extracellular matrix (ECM) that is primarily made of fibronectin, laminin, and collagen[7], and organized in a special three-dimensional architecture with specialized niches[12]. In MM this microenvironment also includes clonal plasma cells that depend for their survival and progression on the signals they receive from this microenvironment. MM cells that express the CXCR4 chemokine receptor, home into the bone marrow by moving along a gradient of the chemokine ligand SDF-1/CXCL12, which is secreted by the bone marrow stroma cells. Upon cell-cell interactions between the MM cells and the bone marrow stroma cells, additional cytokines are induced that promote MM cell proliferation and survival [Figure 1, *e.g.*, IL-6, IGF, TNFα, VEGF, basic fibroblast growth factor (bFGF)][13].

ECs also secrete matrix metalloproteinase-2 (MMP-2) and MMP-9 to help promote their migration, further assisting angiogenesis. Thus, ECs support MM tumor cell survival not only through angiogenesis, but also by promoting cells' invasiveness and dissemination[7].

Thus, MM progression depends greatly on the tumor microenvironment and on the interaction of the tumor cells with the bone marrow stroma cells. However, the exact nature of those interactions, and the identity of all

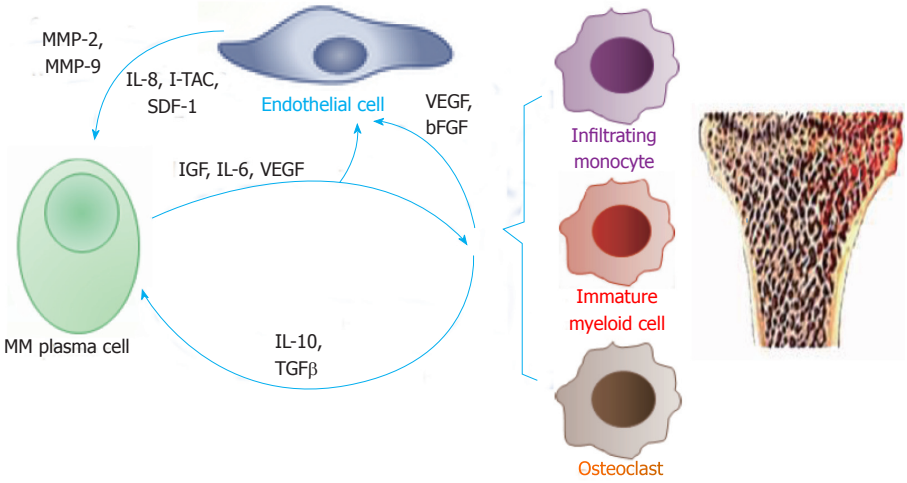

Figure 1 Cell-cell interactions that mediate angiogenesis (in a hypoxic microenvironment). Interactions between multiple myeloma plasma cells, endothelial cells and different myeloid cells (including infiltrating monocytes, immature myeloid cells such as myeloid-derived suppressor cells, and osteoclasts) stimulate secretion of pro-angiogenic factors. MMP: Matrix metalloproteinase; MM: Multiple myeloma; IL: Interleukin; I-TAC: Interferon-inducible T-cell alpha chemoattractant; SDF-1: Stromal cell-derived factor 1; IGF: Insulin-like growth factor; TGFβ: Transforming growth factor beta; VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor.

the proteins mediating them is not yet fully elucidated.

VEGF expression and secretion depend on the local microenvironment

VEGF is highly important to MM progression and viability, due to its critical role in angiogenesis. Many factors collaborate to induce VGEF expression and secretion from MM tumor cells: Both stroma cells and tumor cells secrete IL-6 and IGF-1 that induce VEGF expression, as well as support MM cell growth; local hypoxia contributes to the induction of VEGF *via* the binding of HIF-1α to its hypoxia response elements (HRE) site in the VEGF promoter; adhesion of the tumor cells to the ECM *via* β-integrins also contributes to VEGF induction[7]. Secreted VEGF binds to VEGFR1 on tumor cells and stroma cells to act in both autocrine and paracrine manners. It enhances angiogenesis by promoting EC proliferation and migration, and recruits monocytes and circulatory endothelial precursors to the vasculature, where they may be incorporated into the blood vessels as pericytes or alternatively-activated macrophages. VEGF also enhances the expression of MMP-9[7], that in turn may release VEGF from the ECM, resulting in a positive regulatory loop[14].

The binding of the MM tumor cells to ECs and the binding of VEGF and bFGF to their receptors on ECs activate signaling events that lead to enhanced secretion of chemokines such as CXCL8/IL-8, CXCL11/I-TAC, CXCL12/SDF-1 and CCL-2/MCP-1. These chemokine ligands bind to their receptors on the MM tumor cells, thereby activating and maintaining the paracrine loop between these two cell types, and sustain proliferation and growth of the tumor cells[12]. Additionally, these chemokines, as well as VEGF, are strong chemoattractants of monocytes and macrophages.

The number of bone marrow macrophages increases during active MM, and they can acquire EC-like properties, express EC proteins, and become incorporated into the tumor blood vessels[12]. Moreover, they are an important source of VEGF production by themselves. Involvement of specific macrophage subsets in MM progression and MM-related angiogenesis has been identified within the bone marrow microenvironment. For example, osteoclasts can secrete osteopontin (OPN) and MMP-9, which together with VEGF promote angiogenesis. Myeloid-derived suppressor cells (MDSCs) which are heterologous immature myeloid cells, expand during MM, and exert immunosuppressive effects on the microenvironment, by recruiting regulatory cells (Tregs, more MDSCs and tumor-associated macrophages - TAMs), and by secreting low levels of nitric oxide and immunosuppressive cytokines, such as IL-10 and TGFβ[15,16].

In both mice MM models and human patients, MDSCs expand in the bone marrow, as well as in the spleen and circulation, especially towards the end-stage of the disease, and these bone marrow-derived MDSCs were shown to suppress T cell activity *in vitro*[15]. Additionally, these immature cells can differentiate into macrophages and further into osteoclasts, and MDSCs from MM mice models can differentiate into fully functional osteoclasts *in vitro* and *in vivo*, in higher numbers than MDSCs obtained from naïve mice[15]. Since only MDSCs derived from the bone marrow, but not from the spleen or blood, can undergo this specific differentiation, it is assumed that some specific factor in the bone marrow microenvironment promotes this effect and leads to the generation of bone lesions generated by these osteoclasts. Furthermore, levels of EMMPRIN/CD147, a protein that has been shown to mediate interactions between tumor cells and macrophages, have recently been found to be elevated in plasma

cells (PCs) from MM patients relative to normal PCs. Higher expression levels of EMMPRIN were correlated to increased proliferation of these cells, whereas silencing of the protein reduced their proliferation[17].

Collectively, all the above findings illustrate the importance of the interactions within the bone marrow microenvironment between the different cell types to the regulation of MM pathogenesis in general and MM-related angiogenesis in particular. MM tumor cells elicit stroma cells to produce pro-angiogenic and growth factors that they need for their survival and expansion, while reverse signaling that stroma cells initiate within the tumor cells help sustain these intricate interactions. However, the mechanisms that regulate such interactions require more investigation.

EPIGENETIC REGULATION BY MICRORNA

MicroRNA (miRNAs) are part of a family of non-coding, small (20-25 nucleotides) single-stranded RNA molecules that regulate mRNA translational, stability and degradation. These miRNAs recognize sequences of imperfect complementarity mostly in the 3' untranslated regions (UTRs) of target mRNAs (but also in the 5'-UTR or coding sequences), recruit the RNA-induced silencing complex (RISC) and mediate their translational inhibition. Sometimes miRNAs recognize sequences with perfect complementarity and cause these mRNAs to be degraded[18,19]. More than one miRNA can bind to one transcript at a time, and each miRNA can target hundreds of transcripts, either by binding of their seed sequence (*i.e.*, the 2nd to 7th nucleotides from the 5' side of the mature miRNA) to the 3'-UTR of their target mRNA directly, or by indirectly targeting another transcript that codes for a regulator protein. The details of miRNA biosynthesis are excellently reviewed elsewhere[20-22]. More than 700 human miRNAs have been identified so far, and their role in tumorigenesis and tumor promotion is a subject of intense research. Abnormal changes in the expression of miRNA have been associated with widespread dysregulation of gene expression, inflammation and diverse cancer diseases[23]. Dysregulated expression of miRNA can contribute to tumorigenesis by modulating tumor suppressor genes and oncogene signaling pathways[24]. For example, critical components of key signaling pathways, such as Myc, p53, phosphatase and tensin homolog (PTEN) and NF-κB, are inhibited by miRNAs, leading to the description of miRNAs function as either oncogenes or tumor suppressor genes[24,25].

MM represents a group of diseases that are molecularly distinct. Expression of miRNAs can be dysregulated due to different chromosomal aberrations and genetic mechanisms, or could be regulated directly by proteins involved in the biosynthesis of miRNAs. These miRNAs in turn, regulate the expression of other genes that contribute to the progression and invasiveness of the disease.

METHODOLOGIES USED IN THE STUDY OF MIRNA

In order to associate between a specific miRNA and its target gene, several approaches may be taken. The known sequence of the suspected target gene, and its 3'-UTR in particular, can be searched using different algorithms, which predict the binding of the seed sequence of the miRNA to the target gene[26]. Algorithms, such as those supported by miRNA.org (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), and PicTar (http://pictar.mdc-berlin.de/) are commonly used, but being only predictive tools, they do not necessarily identify real biological sites. Furthermore, these algorithms predict direct binding, but do not address the indirect effects of miRNA, which may affect protein translation through the inhibition of another protein. Therefore, direct binding of the miRNA to the target mRNA must be demonstrated, usually by transfecting tumor cell lines with a reporter construct that contains the 3'-UTR under a luciferase reporter and by measuring the chemiluminescence generated in different conditions.

In order to demonstrate the involvement of a specific miRNA in a biological function, several additional steps are required. First, changes in the expression levels of the specific miRNA could indicate potential regulation, as inhibition of target genes is likely to be affected accordingly. These expression levels are determined by quantitative reverse transcription polymerase chain reaction, and are most commonly normalized to the expression levels of the small nuclear RNAU6 or another stable miRNA that does not change under the experimental conditions. Then the expression levels of the target protein are correlated with the expression levels of the miRNA. Finally, overexpression of the miRNA or its neutralization must be shown to result in a change in the expression of the target protein. These gain or loss of function assays can be performed by transfecting the cells *in vitro* with miRNA mimics or pre-miRNA, or with anti-miRNA (antagomir), respectively. Alternatively, miRNA can be depleted by transfecting the cells with "miRNA sponges", which are plasmid constructs that contain multiple miRNA-binding sites for specific miRNAs under the regulation of a strong promoter, to ensure their high expression[27,28].

INVOLVEMENT OF SPECIFIC MIRNAS IN MM CELL PROLIFERATION AND APOPTOSIS

Dysregulation of miRNA expression, due to chromosomal aberrations and other genetic alterations, may contribute to the dysregulated expression of target genes in MM. In fact, specific miRNA signatures were shown to be associated with specific cytogenetic subgroups in MM[3]. In the remaining parts of this review we will

Table 1 Involvement of microRNAs in multiple myeloma cell proliferation and apoptosis

Activator	miRNA reduced	miRNA overexpressed	Target increased	Target decreased	Effect	Ref.
Deletion, other	miR-15a, miR-16		AKT, S6, MAPK, MAP3KIP3 (TAB3)		Increased proliferation and survival	[30]
	miR-30-5p family		BCL9		Increased Wnt/β-catenin activation	[32]
c-Myc (reduced)	miR-17/92 cluster		Bim		Increased apoptosis	[36,40]
Sp-1 (increased)	miR-29b		Sp-1, CDK6, Mcl-1, caspase-3, Rb phosphorylation			[41,42]
	miR-34a, miR-192, miR-194, miR-215		MDM2, IGF-1		Cell proliferation	[34]
		miR-19a/b		SOCS1, Bim	Reduced apoptosis	[36]
		miR-181a/b, miR-32		PCAF	Reduced p53 acetylation	[36]
		miR-221, miR-222		P27Kip1, p57Kip2, PUMA, PTEN	Increased proliferation	[35]
IL-6		miR-21		STAT-3		[37,38]

AKT: Protein kinase B; MAPK: Mitogen-activated protein kinases; MAP3KIP3: Mitogen-activated protein 3 kinase interacting protein 3; BCL9: B-cell lymphoma 9 protein; MDM2: Mouse double minute 2; IGF-1: Insulin-like growth factor 1; SOCS1: Suppressor of cytokine signaling 1; PCAF: P300/CBP-associated factor; PTEN: Phosphatase and tensin homolog; STAT-3: Signal transducer and activator of transcription 3; PUMA: p53 upregulated modulator of apoptosis; IL-6: Interleukin 6.

describe what is known so far about the involvement of miRNAs in the pathogenesis of MM, focusing specifically on those miRNA that regulate MM-related angiogenesis.

The mechanisms that lead to the dysregulated expression of miRNAs in MM cells are not always fully elucidated. In some cases, the malignant transformation itself, *i.e.*, translocation of chromosomal fragments or deleted chromosome segments, could lead to either reduction or complete loss of some miRNA's expression, or to enhanced expression of other miRNAs that could down-regulate the expression of a protein needed for the transcription of another miRNA. Indeed, a correlation between deregulated miRNA expression and cytogenetic abnormalities in MM cells was recently found[29]. In other cases, DNA methylation of promoters that encode for miRNAs was found, resulting in their silenced expression[13]. Thus, different mechanisms could lead to aberrant expression of miRNAs and their involvement in MM pathogenesis.

Many of the miRNAs that were shown to contribute to MM pathogenesis actually regulate MM cell proliferation and/or apoptosis (summarized in Table 1). Central to these regulatory loops are miR-15a and miR-16 that are located as a cluster on chromosome 13q14, an area commonly deleted in MM. Thus, patients with deletion in chromosome 13, which make up about 50% of MM patients, also exhibited total lack of miR-15a and miR-16[30]. In patients whose chromosome 13 is not deleted, miR-15a and miR-16 expression is often reduced, and is inversely correlated with advanced stage of the disease[31]. The reduced expression levels of miR-15a and miR-16 regulate proliferation and growth of MM cells both *in vitro* and *in vivo* in the bone marrow microenvironment, by inhibiting several target proteins, such as the AKT serine/threonine protein kinase (AKT3), ribosomal-protein-S6, MAP kinases, and the NF-κB activator MAP3KIP3 (TAB3)[30]. Specifically, NF-κB activation plays a pivotal role in promoting growth and survival of MM cells, and is regulated by the interaction of MAP3KIP3 with TAK1. When MM cells were transfected with pre-miR-15a and pre-miR-16-1, MAP3KIP3 protein expression levels were reduced, and consequently, TNFα-activation of the NF-κB family of proteins p65, p50 and p52, that normally results in their recruitment into the nucleus, was inhibited, whereas phosphorylated IκB was increased in the cytoplasm. Thus, MAP3KIP3 is a validated target gene of miR-15a and miR-16[30].

The family of miR-30-5p (including miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, and miR-30e-5p) is down-regulated in plasma cells derived from MM patients, compared to normal cells. Since this family targets BCL9, a critical transcriptional coactivator of β-catenin, its absence causes the Wnt/β-catenin pathway to be over-active, and promotes MM cell proliferation, survival, migration, drug resistance,

and formation of MM cancer stem cells[32]. In fact, the Wnt/β-catenin pathway is constitutively active in MM, promoting tumor cell proliferation, and resistance to chemotherapy[33].

Involvement of wild type p53 in MM pathogenesis is also regulated by the down-regulation of miR-34a, miR-192, miR-194 and miR-215 in some MM patients and cell lines, which is caused by the hyper-methylation of their respective promoters. Reduced levels of these miRNAs that directly target MDM2, the negative regulator of the p53, disrupt the balance between MDM2 and p53, and favors MDM2 stability. In contrast, overexpressing these miRNAs in MM cells carrying the wild type TP53 results in growth arrest[34].

Overexpression of other miRNAs creates similar effects, and several examples illustrate the complementary role they play in the regulation of cell proliferation and apoptosis. Proliferation of MM cells is partly regulated by miR-221/222, and some MM cells, such as the TC2 and TC4 subtypes that share the t(4:14) translocation, highly express miR-221/222. The neutralization of miR-221/222 in these MM cells reduced proliferation and up-regulated the expression of their target genes p27Kip1, PUMA, PTEN, and p57Kip2[35]. In another study, overexpression of miRNA-222, miR-221, miR-382, miR-181a and miR-181b was found in MM $CD138^+$ cells in comparison to normal cells, without assigning to them specific functions. However, their targets were predicted to be tumor suppressors, cytokine signaling suppressors (SOCS1, SOCS6), pro-apoptotic factors, NF-κB suppressors, and tyrosine phosphatases[30]. In support of these results, another study found overexpression of miR-32, miR-181a, miR-181b and also miR-21 that were demonstrated to target the p300-CBP-associated factor, that positively regulates p53 by acetylation[36].

IL-6 is essential as a growth factor for B cells and can also up-regulate miR-21 expression through activation of STAT-3, which has two binding sites in the miR-21 promoter. Thus, ectopic overexpression of miR-21 could sustain growth of MM cells, even in the absence of IL-6[37]. Moreover, a positive feedback loop exists, as miR-21 inhibits the expression of the protein inhibitor of activated STAT3, leading to enhanced STAT3 signaling[38]. In patients or human MM cell lines that are intrinsically resistant or develop resistance over time to treatment with bortezomib, elevated levels of miR-21 can be found, whereas in responding cells or patients, bortezomib reduces miR-21 levels[39]. Thus, miR-21 may become an attractive target for enhancing treatment efficiency in MM patients. Additionally, miR-19a and miR-19b were also found to be overexpressed, and were shown to inhibit the expression of SOCS1 and of the pro-apoptotic protein BIM/BCL2L11[36].

Expression of the c-Myc oncogene can be dysregulated in many MM patients, due to chromosomal translocations or other mechanisms. Silencing of c-Myc in MM cell lines leads to reduced cell proliferation and triggers apoptosis, as well as inhibited expression of the miR-17-92 cluster, demonstrating that c-Myc positively regulates this cluster. Triggering of apoptosis in these cells was the result of strong activation of the pro-apoptotic Bim protein, whereas the anti-apoptotic Bcl2 or Bcl-xL proteins were unaffected. Overexpression of miR-17 and miR-18, that belong to this cluster, even in the absence of c-Myc, inhibited Bim expression. Thus, Bim is directly regulated by the miR-17-92 cluster, which is in turn, activated by c-Myc, and these results implicate the cluster in the process of apoptosis[40].

In MM patients and MM cell lines the expression of miR-29b is down-regulated. Overexpression of miR-29b inhibits cell growth and induces apoptosis in MM cells, partly by directly targeting the anti-apoptotic protein Mcl-1 and by activating caspase-3[41]. Additionally, the anti-proliferative and pro-apoptotic properties of miR-29b are partly exerted by its inhibitory effects on Sp1 expression. On the other hand, Sp1 regulates miR-29b transcription, generating a negative feedback loop between the two factors. The proteasome inhibitor bortezomib affects this miR-29b-Sp1 loop by decreasing Sp1 and elevating miR-29b expression. Likewise, PI3K/AKT is also involved in the regulation of this balance between the two factors, as it works as a negative regulator of miR-29b expression[42].

SPECIFIC MIRNAS TARGETING MEDIATORS OF ANGIOGENESIS IN MM

Angiogenesis plays a crucial role in the pathogenesis and progression of MM, and greatly depends on the interactions of MM cells with the stroma cells, particularly endothelial cells and macrophages. These interactions may lead to changes in the expression of different miRNAs and consequently to regulation of their target gene expression (Figure 2).

The key cells involved in angiogenesis are the endothelial cells, and to promote angiogenesis, MM tumor cells must support their proliferation and migration by releasing VEGF in a paracrine manner. VEGF mediates vascular permeability and induce endothelial cell growth and migration to allow angiogenesis, invasiveness and metastasis. It is also a chemoattractant to macrophages and a regulator of MMP-9, thus it can also indirectly enhance angiogenesis[43]. Therefore, VEGF is perhaps the most potent pro-angiogenic factor known, and its enhanced expression, along with other pro-angiogenic factors, is regulated at several check points including by miRNAs.

First, hypoxia has been shown to induce VEGF expression by the binding of the HIF-1α transcription factor to its binding site (HRE) on the VEGF promoter, as well as to other pro-angiogenic factors such as angiopoietin-2, MMPs, and semaphoring 4D. Local and chronic hypoxia is generated in the bone marrow due to the increasing metabolic needs of proliferating MM cells. Although the bone marrow is naturally hypoxic, studies show that the oxygen tension levels in MM

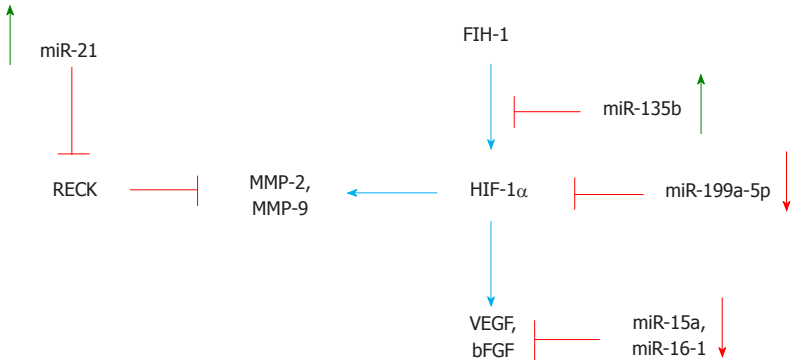

Figure 2 Key mediators of angiogenesis in multiple myeloma and their regulation by miRNAs. Pro-angiogenic factors are subject to regulation by hypoxia that triggers hypoxia-induce factor 1α, and by other signals (*e.g.*, TGFβ, TNFα), and are fine-tuned by different microRNAs. Green arrows, increased expression levels; red arrows, decreased expression levels. RECK: Reversion-inducing-cysteine-rich protein with kazal motifs; MMP: Matrix metalloproteinase; FIH-1: Factor inhibiting HIF-1; HIF-1α: Hypoxia-inducible factor 1-alpha; VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor.

bone marrow are even lower[44]. This prolonged hypoxic microenvironment exerts pressure on the malignant cells, and those surviving MM cells, which become hypoxia-resistant, were shown to secrete twice the amount of miR-135b-containing exosomes. These exosomes were up-taken by endothelial cells, and their cargo of miR-135b directly targeted the factor inhibiting HIF-1, which inhibits HIF-1α activity. Thus, prolonged, but not acute hypoxia, can mediate interactions between MM tumor cells and endothelial cells to elevate angiogenesis[45]. However, it should be remembered that hypoxic exosomes may contain additional miRNAs, in additional to miR-135b, that might work cooperatively with miR-135b to regulate angiogenesis[46]. Hypoxia also works through the down-regulation of miR-199a-5p expression, which directly targets HIF-1α. Thus, hypoxic MM cells up-regulate HIF-1α and through it induce the expression of several pro-angiogenic factors, such as VEGF, IL-8, bFGF, and CXCL-12/SDF-1, whereas MM cells transfected with synthetic miR-199a-5p showed reduced expression of these factors[47]. Conditioned medium obtained from such cells and incubated with human umbilical vein endothelial cells (HUVEC) caused their reduced migration and inhibited production of VEGF, VCAM-1, ICAM-1 and IL-8[47], again demonstrating the importance of tumor-stroma cells interactions for induction of angiogenesis.

Secondly, VEGF and bFGF are predicted target genes for miR-15a and miR16. Accordingly, MM cell lines that were transfected with pre-miR-15a and pre-miR-16-1 demonstrated reduced secretion of VEGF[30,31], and conditioned medium from these cells reduced the activation of the ERK or AKT pathways in endothelial cells, resulting in their reduced ability to form tube-like formations *in vitro*[30].

Enzymes that are involved in the biosynthesis of miRNAs also indirectly regulate angiogenesis, by affecting specific miRNAs expression. For example, argonaute 2 (Ago2) is a core component of the RISC. Supernatants from Ago2-overexpressing MM cell lines induced HUVEC migration and accelerated tube formation, whereas supernatants from Ago2-knockdown MM cell lines suppressed HUVEC cell migration and tube formation[48]. This effect was mediated through Ago2-driven up-regulation of 25 miRNAs (including members of the let 7 family and the miR-17/92 cluster) and down-regulation of 7 miRNAs (including miR-145 and miR-361). The 17/92 cluster includes miR-92-1, which was up-regulated by Ago2, targeted the angiopoietin-like protein 1 (ANGPTL1, an anti-angiogenic protein with tumor-inhibiting properties), and down-regulated its expression[48]. Overexpression of Ago2 in MM cells also resulted in the down-regulation of miR-145, which directly targets VEGF, and therefore, increased VEGF expression[48] (Table 2).

Lastly, in MM cells, miR-21 is upregulated, in reverse correlation to the down-regulated expression of reversion-inducing-cysteine rich protein with kazal motifs (RECK), a protein inhibitor of MMP-2 and MMP-9[49]. Hypoxic MM cells show increased levels of MMP-2 expression, whereas enforced expression of miR-199a-5p in these cells reduced MMP-2 levels[47].

USING MIRNAS AS BIOMARKERS OF MM AND AS POTENTIAL THERAPY FOR MM

Attempts have been made to use miRNA expression profiles as biomarkers for MM progression, or for classification of the MM cells into specific cytogenetic subtypes. Some miRNA arrays have been used to identify specific signatures or miRNA profiles that characterize different stages of MM progression and differentiate between MGUS and symptomatic MM[3,36]. Since miRNAs are involved in MM pathogenesis and regulate many of the molecular processes that dictate the course of the disease, it is reasonable to assume that miRNA profiling or determination of expression of specific miRNA may have diagnostic and/or prognostic value. Given their reported stability in serum[50], miRNA expression may represent novel non-invasive biomarkers of MM. This seems a promising direction for further study.

Table 2 Involvement of microRNAs in the regulation of multiple myeloma-related angiogenesis

	Target increased	Target reduced	Effect (anti-angiogenic)	Effect (pro-angiogenic)	Ref.
miRNA reduced					
miR-15a, miR-16, miR-145	VEGF, ERK pathway, AKT pathway		Reduced EC growth, reduced ability to form capillary structures		[30,31,48]
miR-199a-5p	HIF-1α, SIRT-1		Increased expression of VEGF, IL-8, bFGF, CXCL12, MMP-2		[47]
miRNA increased					
Let 7 family		Thrombospondin-1, TIMP-1		Inhibiting anti-angiogenic factors	[48]
miR-92-1		Angiopoietin-like protein 1		Inhibit anti-angiogenic protein	[48]
miR-135b		FIH-1		Alleviate HIF-1α inhibition	[45]

VEGF: Vascular endotelial growth factor; ERK: Extracellular signal-regulated kinase; HIF-1α: Hypoxia inducible factor 1 alpha; SIRT-1: Sirtuin 1; FIH-1: Factor inhibiting HIF-1; IL-8: Interleukin-8; bFGF: Basic fibroblast growth factor; MMP: Matrix metalloproteinase.

Furthermore, the dysregulated expression of miRNAs places them as novel candidate therapeutic targets. Because miRNAs simultaneously target the expression of several genes and regulate key signaling pathways, targeting them is likely to be more beneficial than conventional approaches targeting a single protein with a single drug[51]. The problem of delivering small RNA molecules to tumor cells within the bone marrow without using viral vectors, and then making sure that the miRNAs or antagomirs are taken up specifically by the tumor cells has been addressed by developing lipid-based or polymer-based delivery systems[51]. Another possible advantage of using miRNA for therapy is the relative ease of detecting aberrant expression of specific miRNAs in the serum of MM patients or even in their bone marrow, and the ability to closely follow up on changes of miRNAs expression in response to treatment. Collectively, these advantages may promote, in the future, a personalized medicine approach, where patients will be specifically-tailored with antagomirs or miRNA mimics according to their personal miRNA expression profile, hopefully increasing the success of the treatment.

Currently, there are only a limited number of studies demonstrating the efficiency of targeting miRNAs as therapeutic means in MM, mostly carried out as pre-clinical trials using immunocompromised mice. One study introduced a mix of miRNAs from the miR-30 family, or just miR-30c, into MM cells, and observed reduction in tumor burden and in metastases in three human MM xenograft models, without adverse effects such as bone lesion. This effect was attributed to the ability of the miR-mimics to reduce and keep in check the expression of BCL9, the transcriptional coactivator of the Wnt signaling pathway[32]. Overexpression of miR-199a-5p in human MM NCI-H929 xenograft in NOD/SCID mice was achieved by six intra-tumoral injections, every 3 d, of 20 μg each encased in neutral lipid emulsion. This treatment reduced tumor growth and prolonged survival of the treated mice in comparison to the control mice[47]. The only example so far, to the best of our knowledge, which targets MM-related angiogenesis is the injection of miR-15a and miR-16 to the tail vein of mice bearing *s.c.* human MM xenografts, where reduced tumor size was observed due to reduced angiogenesis[31].

CONCLUSION

MiRNA have a key role in MM, regulating cellular processes that are essential to disease initiation and progression. Most studies in the field focus on the involvement of miRNA in the regulation of tumor cell proliferation, survival, and apoptosis. Only a limited number of studies investigate the involvement of miRNAs in the regulation of angiogenesis, a process that is critical to disease progression and especially to the malignant transformation from MGUS to MM. Thus, manipulation of this process represents a promise to attenuate the progression of the tumor to the malignant stage. Furthermore, the novel drugs, such as thalidomide, lenalidomide and bortezomib have been shown to exert an anti-angiogenic effect in MM patients. Understanding how they regulate miRNAs may lead to better treatment approaches by fine-tuning the drugs' properties to manipulate specific miRNAs expression. We therefore anticipate and hope that the following years will lead to exciting new investigations into the involvement of miRNA in MM pathogenesis, and specifically in MM-related angiogenesis, studies that will hopefully be implemented in treatment of this still incurable disease.

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REVIEW

Changing insights in the diagnosis and classification of autosomal recessive and dominant von Willebrand diseases 1980-2015

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Abstract

The European Clinical Laboratory and Molecular (ECLM) criteria define 10 distinct Willebrand diseases (VWD): recessive type 3, severe 1, 2C and 2N; dominant VWD type 1 secretion/clearance defect, 2A, 2B, 2E, 2M and 2D; and mild type 1 VWD (usually carriers of recessive VWD). Recessive severe 1 and 2C VWD are characterized by secretion and multimerization defects caused by mutations in the D1-D2 domain. Recessive 2N VWD is a mild hemophilia due to D'-FVIII-von Willebrand factor (VWF) binding site mutations. Dominant 2E VWD caused by heterozygous missense mutations in the D3 domain is featured by a secretion-clearance-multimerization VWF defect. Dominant VWD type 2M due to loss of function mutations in the A1 domain is characterized by decreased ristocetin-induced platelet aggregation and VWF:RCo, normal VWF multimers and VWF:CB, a poor response of VWF:RCo and good response of VWF:CB to desmopressin (DDAVP). Dominant VWD type 2A induced by heterozygous mutations in the A2 domain results in hypersensitivity of VWF for proteolysis by ADAMTS13 into VWF degradation

products, resulting in loss of large VWF multimers with triplet structure of each individual VWF band. Dominant VWD type 2B due to a gain of function mutation in the A1 domain is featured by spontaneous interaction between platelet glycoprotein Ib (GPIb) and mutated VWF A1 followed by increased proteolysis with loss of large VWF multimers and presence of each VWF band. A new category of dominant VWD type 1 secretion or clearance defect due to mutations in the D3 domain or D4-C1-C5 domains consists of two groups: Those with normal or smeary pattern of VWF multimers.

Key words: Von Willebrand disease; Von Willebrand factor; ADAMTS13; DDAVP; Von Willebrand factor assays; Von Willebrand gene mutations

Core tip: The European Clinical Laboratory and Molecular criteria define at least 10 distinct phenotypes of von Willebrand diseases (VWD) that have significant therapeutic implications. High quality von Willebrand factor (VWF) multimeric analysis and responses to desmopressin of FVIII:C and VWF parameters are of critical diagnostic importance to document the contribution of VWF secretion, clearance, proteolysis and multimerization defects to real life phenotyping of each individual VWD patient.

Michiels JJ, Batorova A, Prigancova T, Smejkal P, Penka M, Vangenechten I, Gadisseur A. Changing insights in the diagnosis and classification of autosomal recessive and dominant von Willebrand diseases 1980-2015. *World J Hematol* 2016; 5(3): 61-74 Available from: URL: http://www.wjgnet.com/2218-6204/full/v5/i3/61.htm DOI: http://dx.doi.org/10.5315/wjh.v5.i3.61

INTRODUCTION

Von Willebrand factor (VWF) is biosynthesized exclusively in vascular endothelium and megakaryocytes. The precursor protein proVWF consists of a signal peptide (22 amino acids, aa), the propeptide (741 aa) and the mature VWF monomer (2050 aa) (Figure 1)[1,2]. The intracellular uncleaved VWF (2791 aa) has 14 distinct domains from left to right: D1, D2, D', D3, A1, A2, A3, D4, B1-3, C1, C2 and CK (Figure 1). The exons which encode each domain are shown in Figure 1 above the VWF domain. The areas of VWF involved in binding specific functional factors are shown in Figure 1 below the VWF domains[1,2]. During the translocation of proVWF to the endoplasmatic reticulum the signal peptide is cleaved off, and the proVWF forms dimers in a tail-to-tail fashion through cysteines in its carboxyterminal cysteine knot (CK) domain (Figure 2)[3-5]. ProVWF dimers transit to the Golgi apparatus as multimers through disulphide bonds between cystein residues in the D1-3 multimerization domain. Meanwhile, the D1-D2 domains are cleaved off to form the VWF propeptide (VWFpp, 741 aa), while the remaining domains from D' to CK form mature VWF (2050 aa, Figures 1 and 2). In the trans Golgi network, VWFpp promotes high molecular weight multimer formation in tubular structures, subsequently packaged in Weibel Palade bodies (WPB)[3-5]. When the endothelium is exposed to certain stimuli such as desmopressin (DDAVP), WPB undergo exocytosis and release their contents into the circulation or present them on the cell surface as string-like structures[3,4]. These high molecular weight VWF recruit platelets from the circulating blood to bind, upon which the ultralarge VWF are cleaved into the normal spectrum of high, intermediate and small strings (multimers) by the VWF cleavage protease ADAMTS13 at high shear stress in the endarterial circulation (Figure 2)[4,5]. At the time that VWF is secreted from WPB in the endothelial cell, the VWF propeptide (VWFpp = D1D2 domain) is cleaved off again at the furin cleavage site (Figure 3). Mutations in the D1 and D2 domains mean that the propeptide VWF cannot cleave off from the mature VWF, with the consequence of a VWF secretion and multimerization defect, explaining the loss of large VWF multimers in recessive severe type 1 and 2C disease (Figure 3).

VWF-FVIII and VWF-platelet interactions

VWF circulates as a multimeric plasma glycoprotein with coagulation factor VIII (FVIII:C) bound to the D' domain of VWF[6]. FVIII is cleaved off from VWF by thrombin at sites of vascular injury. VWF circulates as large multimers as a function of the D3 multimerization and CK-terminal dimerization domains. Activated VWF and platelets mediate platelet adhesion to subendothelium and platelet aggregation at sites of vascular injury (Figure 4)[6]. At sites of vascular injury and high shear, activated platelets and activated VWF aggregate through binding of platelet GpIb to the VWF A1 domain. In the equilibrium state, with intact endothelial cells and no injured blood vessel, resting VWF circulates in globular form with resting platelets in the blood (Figure 4A). In this state, VWF is incapable of mediating platelet adhesion. After an injury of the endothelial cells, the activated and elongated VWF interacts with exposed collagen *via* VWF domains A1 and A3 and triggers the adhesion of activated platelets *via* VWF GPIb, collagen binding and GPIIb/IIIa domains (Figure 1). At low shear there is no binding between VWF domain A1 and platelet GPIb. At high shear rate the VWF globules elongate and make the VWF A1 domain accessible by the dissociation of domain A1 from A2 (Figure 4). Binding between GPIb of activated platelets to the GPIb receptor of VWF is immediately followed by cleavage of VWF in the A2 domain by ADAMTS13 (Figure 4).

Von Willebrand disease type 1, 2 and 3

The introduction of ristocetin-based assays VWF:RCo and ristocetin induced platelet aggregation (RIPA), and the

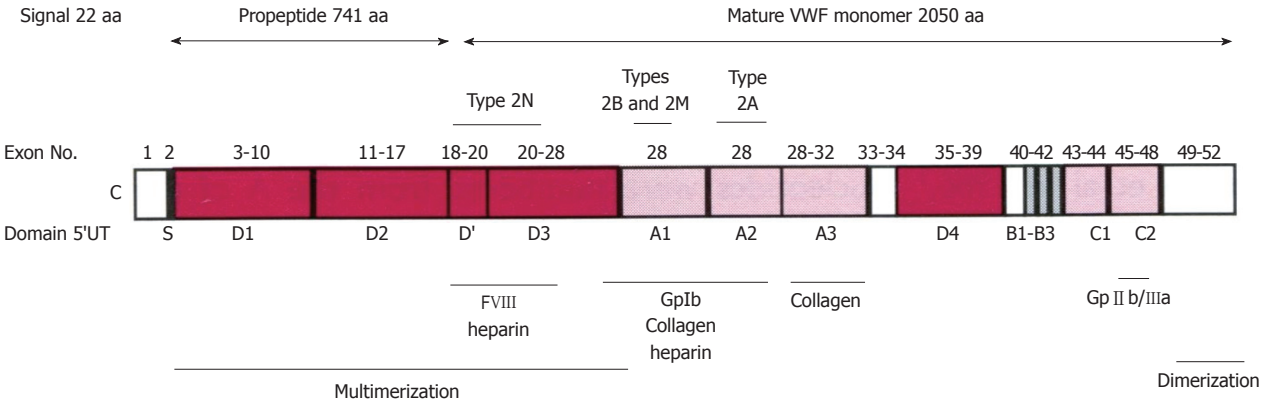

Figure 1 Structure and function relationship of the von Willebrand factor domains[1]. The VWF is synthesized in endothelial cells as a large protein of 2813 amino acid (aa): signal prepetide 22 aa, propeptide 741 aa, and the mature VWF monomer 2050 aa. D1-D2 pro-peptide is cleaved off at the furin cleavage site at time of secretion. VWF circulates bound to the FVIII at the D' FVIII binding domain. Below the figure are the areas of VWF involved in binding specific factors. VWF circulates as large multimers as a function of the D3 multimerization and CK dimerization domains. Source: Goodeve and Peake[1]. VWD: Von Willebrand disease; VWF: Von Willebrand factor.

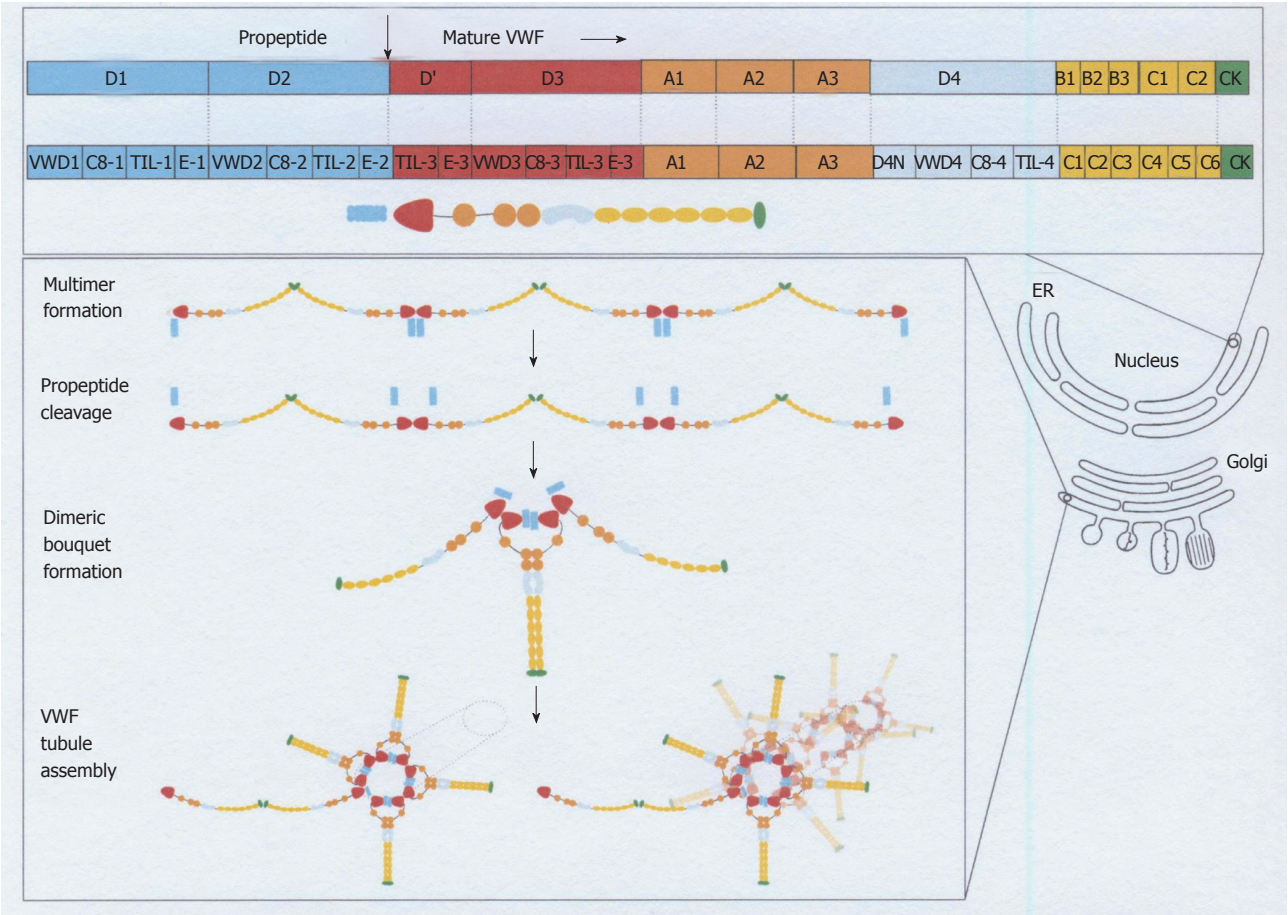

Figure 2 Von Willebrand factor domain structure and assembly throughout the biosynthetic pathways in endothelial cells[3-5]. The top panel shows the different domains of VWF as it is synthesized in the ER[4]. The arrow between the D2 domain and the D' domain indicates the furin cleavage site at 764 leading to the production of the VWF propeptide (VWFpp) D1-D2 (blue) and the mature VWD protein with the domains D', D3, A1, A2, D4, C1-6 and the cysteine knot (CK). The lower panel shows the assembly of VWF into multimers in the Golgi compartment, the cleavage VWFpp (blue), and the assembly of VWF into the dimeric bouquet at the trans-Golgi network (TNG). During the translocation of proVWF to the ER the signal peptide is cleaved off, and the proVWF forms dimers in a tail-to-tail fashion through cysteines in its carboxyterminal cysteine knot domain. ProVWF dimers transit to the Golgi apparatus to assemble into multimers in a "head-to-head" fashion through the formation of intermolecular disulphide bonds between cysteine residues in the D3 (multimerization) domain[4]. This is followed by the assembly of VWF in the Golgi network. ER: Endoplasmatic reticulum; VWD: Von Willebrand disease; VWF: Von Willebrand factor. Source: Valentijn and Eikenboom 2013[4].

VWF:RCo to FVIIIR:Ag (VWF:Ag) ratio, combined with VWF multimeric analysis in the 1970s were the first steps in the classification of von Willebrand disease (VWD)[7,8]. In 1973, Firkin *et al*[7] discovered increased RIPA at low

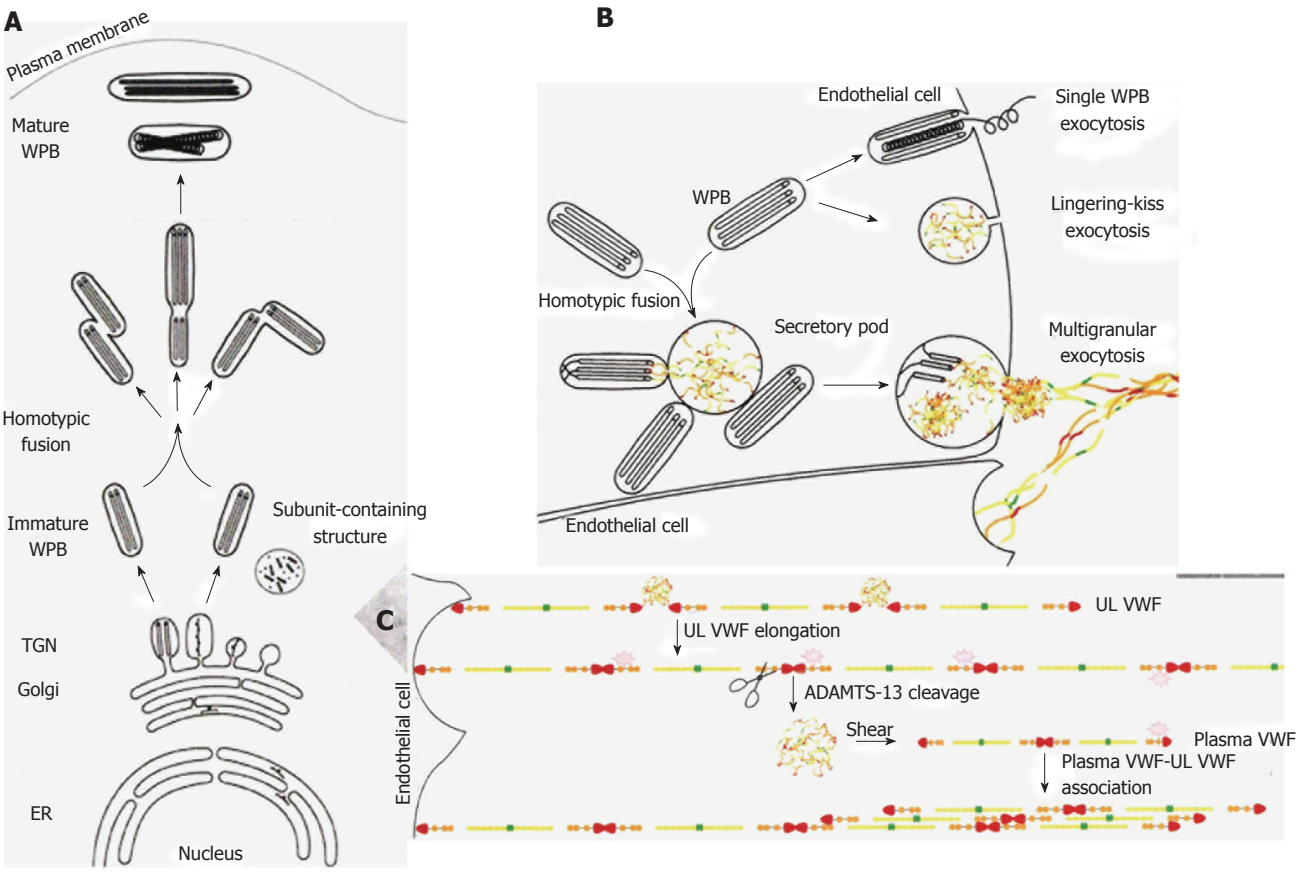

Figure 3 A left biosynthesis pathway of Weibel-Palade body[4]. A, B: The different steps in WPB synthesis of von Willebrand factor (VWF) assembly at the level of endoplasmatic reticulum (ER), at the trans-Golgi network (TGN) level (A), and VWF tubules are assembled and packed into budding vesicles prior to immature WPB formation. Homotypic fusion of WPB gives rise to the formation of WPB with different shapes. As WPB mature they became more electron-dense and reach the plasma membrane. B: Different modes of WPB exocytosis and VWF string formation on endothelial cells. In single WPB exocytosis mode, a single WPB fuses with the plasma membrane and ultra large VWF multimers (MM) are secreted. In lingering-kiss exocytosis mode (B), WPB round up and a small pore is formed with the plasma membrane, allowing the secretion of ultra large VWF MM. In multigranular exocytosis mode (B), WPB undergo homotypic fusion leading to the formation of a secretory pod that permits pooling of ultra large VWF MM prior to secretion[4]. After release, the ultra large vWF strings stick to the endothelial cell surface, attract platelets through platelet GpIb ligand and VWF GpIb receptor interaction, thereby activating the VWF cleavage site to be cleaved by ADAMTS13 at high shear stress in the endarterial circulation (C). WPB: Weibel-palade body. Source: Valentijn and Eikenboom 2013[4].

ristocetin concentrations as a pathognomonic finding for VWD type IIB as a distinct bleeding diathesis. Ruggeri *et al*[8] confirmed the association of heightened interaction between platelets and VWF in type IIB VWD. In contrast, RIPA was decreased or absent in type IIA VWD. The 1986 Zimmerman Classification of VWD[9] could distinguish five main variants of type 2 VWD: IIA, IIB, IIC, IIE and IID (Figure 5). Loss of large VWF multimers due to increased proteolysis into 176 kDa and 140 kDa degradation products is seen in VWD type IIA and IIB. In contrast, proteolytic VWF fragments (degradation products) are absent in VWD type IIC, IIE and IID as compared to VWF multimers in normal plasma[2,9,10]. Consequently, the loss of large VWF multimers in VWD 2C and 2E is not due to increased proteolysis, but caused by a multimerization defect due to mutations in the D1-D2 and D3 domains (Figures 6 and 7)[3,11,12].

Three main categories of VWD can be distinguished: firstly, a category of recessive type 3, severe type 1 and 2C; secondly, a category of dominant type 1 and 2, and thirdly, large category of mild VWD with no or low penetrance of bleeding manifestations[12-20]. Recessive VWD type 3, a hemophilia-like bleeding disorder with a complete absence of VWF and FVIII is caused by a homozygous or double heterozygous non-sense mutation in the VWF gene[21-23]. Recessive severe "type 1" VWD differs from "type 3" VWD by double heterozygosity for a non-sense/missense or two missense mutations with the presence of detectable VWF:Ag and FVIII:C levels between 0.09 and 0.40 U/mL[24-33]. Double null mutations in recessive type 3 VWD are distributed over all domains and exons of the VWF gene. Missense mutations causing recessive severe type 1 are mainly located in the exons 3 to 11 of the D1-D2 domains (*e.g.*, *D47H*, *S85P*, *Y87S, D141Y*, *D141N*, *C275S*, *W377C, I427N)*, and in exons 36 to 52 of the D4, B1-3, C1-2, CK domains (*e.g.*, *P2063S*, *C2174G*, *C2362F*, *N2546Y*, *C2671Y*, *C2754W and C2804Y*)[24-33].

The 2N mutations *E787K*, *T791M* and *R816W* cause a severe type 2N phenotype with less than 10% FVIII binding (FVIIIB) to VWF. Homozygous or double heterozygous R854Q mutations are the most frequent

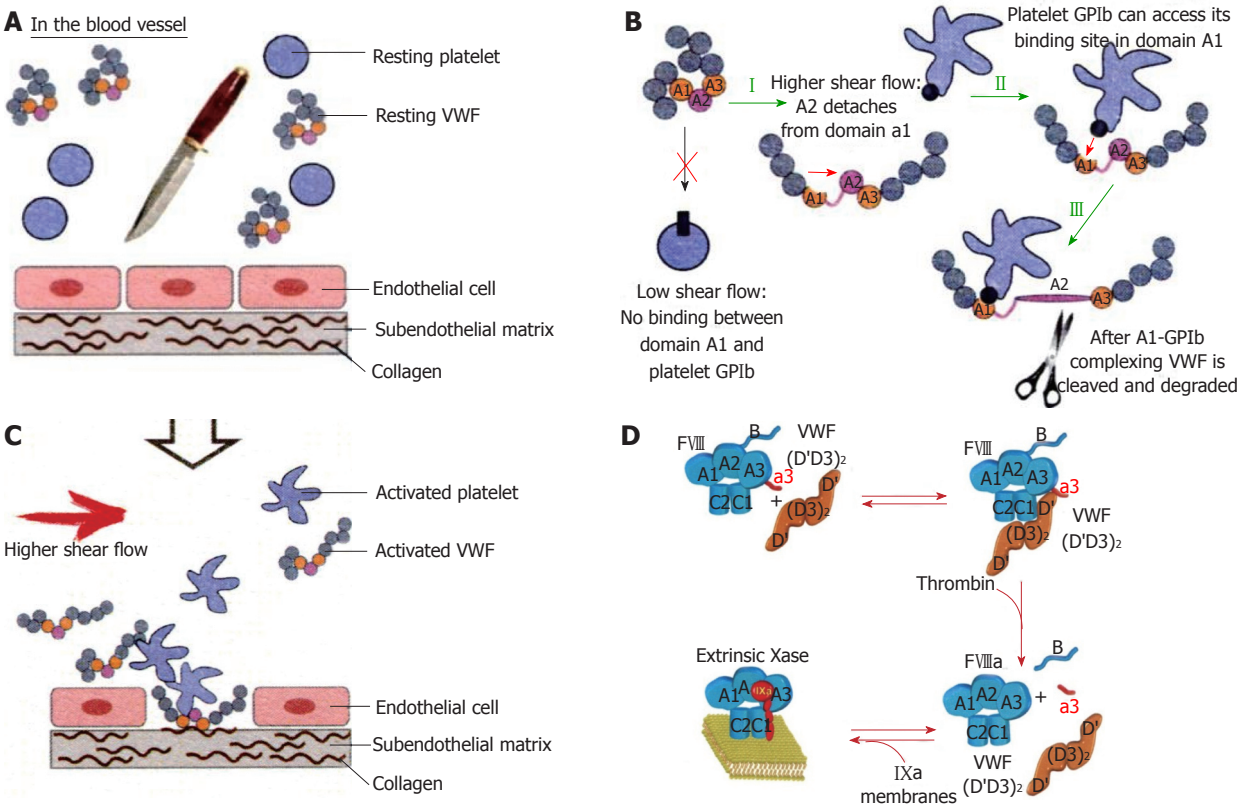

Figure 4 In the equilibrium state, with intact endothelial cells and no injured vessel, resting von Willebrand factor circulates as globules with resting platelets in blood (A). In this state, VWF is incapable of mediating platelet adhesion. After an injury of the endothelial cells, the activated VWF: Von Willebrand factor (VWF) interacts with exposed collagen *via* vWF domains A1 and A3 (orange parts) and triggers the adhesion of activated platelets *via* VWF domain A1 (B, C). At low shear there is no binding between VWF domain A1 and platelet GPIb. At high shear rate the VWF globules elongate and made the VWF A1 domain accessible by the dissociation of domain A1 from A2. High shear flow detaches the A2 domain from domain A1 (I). Binding between GPIb of activated platelet to the GPIb receptor of VWF (II), which is immediately followed by cleavage of VWF in the A2 domain by ADAMTS13 (III) (B). Courtesy of Dr Sandra Posch. Insitute of Biophysics. Linz, Austria: sandra.posch@jku.at. FVIII:C is a heterodimer with a domain structure of A1-A2-B-A3-C1-C2 (upper left, Blue). FVIII:C circulates in complex with VWF through binding to the D'D3 domain, the FVIIIbinding site on VWF. Thrombin cleavage of FVIII liberates the a3 peptide and the B domain of FVIII (D), resulting in the dissociation of VWF from FVIII[6].

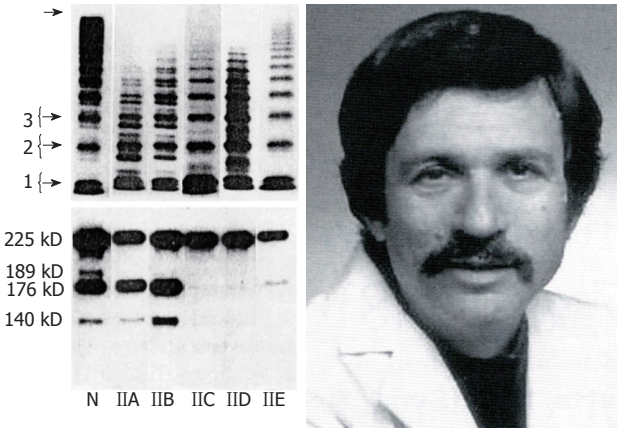

Figure 5 The 1986 Zimmerman Classification of Von Willebrand disease type IIA, IIB, IIC, IID and IIE[9] and Dr. Ted Zimmerman (1937-1988). SDS-agarose multimeric analysis of plasma VWF in normal plasma (N) and in VWD type IIA, IIB, IIC, IIE and IID. Left lower part: Immunoblots of VWF proteolytic degradation products show increased proteolysis in VWD type IIA and IIB, but not or even absent in VWD type IIC, IIE and IID (N = normal plasma). VWD: Von Willebrand disease; VWF: Von Willebrand factor.

findings in type 2N and are associated with mild FVIII binding defects of around 25%[6,34,35]. A normal multimer distribution is observed in non-cysteine mutated VWD 2N patients in whom bleeding episodes are similar to those in patients with mild/moderate hemophilia A, with bleedings occurring after trauma or surgery. Type 2N mutations that involve a cysteine (*C788R/Y*, *Y795C*, *C804F* and *C858S/F*) are associated with aberrant multimerization, poor secretion and reduced FVIII binding[34,35]. Three mutations (*T791M*, *R816W* and *R845W*) account for the majority of typical 2N cases with normal VWF multimers[33,34]. Patients with mild 2N VWD (*e.g.*, homozygous *R854W*) can be treated for minor bleeds by DDAVP administration[18,35]. Obligate carriers of recessive type 3, recessive severe type 1 and recessive 2N VWD are heterozygous for a non-sense (null) or missense mutation, and are usually asymptomatic at VWF levels around 50 U/mL[16,32,33].

Translation of VWD IIC, IIE, IIA, IIB and IID into 2C, 2E, 2M, 2A, 2B and 2D

The International Society on Thrombosis and Haemostasis (ISTH) classification of VWD is based on 5 relatively "insensitive" laboratory tests (FVIII:C, VWF:Ag, VWF:RCo, RIPA and VWF multimers in low resolution gels) (Table 1)[13-15]. The ISTH criteria cannot clearly

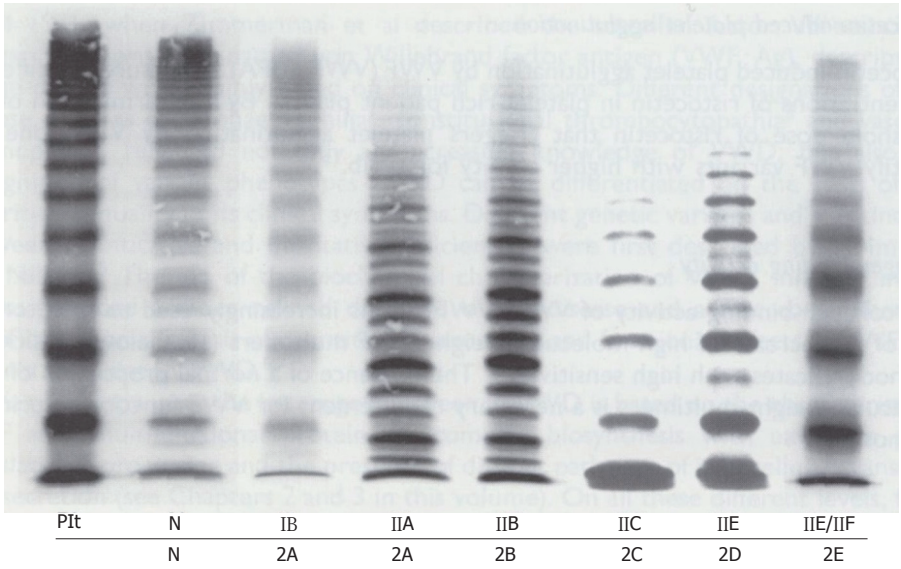

Figure 6 Multimeric pattern. VWF from plasma of patients with von Willebrand disease classified according to the ISTH criteria for VWD type IIA, IIB, IIC and IIE and the translation into the 2001 Hamburg criteria for 1B = 2M (not 2A), 2A, 2B, 2C, 2D and 2E anno 2001 compared to a normal control in high resolution gel concentration (1.5%) according to Schneppenheim *et al*[10]. Dominant 1B relatively lacking large VWF multimers (MM) = 2M (Michiels). Dominant IIA = 2A lack of large molecular weight MM and the outer sub-bands of the individual triplets are markedly pronounced indicating increased proteolysis as the cause of 2A. Dominant 2B cannot be distinguished from 2A by MM alone. Recessive IIC = 2C lack of large MM and absence of triplets. Low MM and especially the first band, which probably reflects protomer (dimer) and a tetramer, is markedly pronounced. IID = 2D intervening VWF band and an odd number of MM. 2E lack or relative decrease of large MM and absence of the outer sub-bands of the normal triplet structure. Triplets are lacking in 2C, 2D, 2E and 1B = 2M are lacking indicating the absence of proteolysis. VWD: Von Willebrand disease; VWF: Von Willebrand factor. Plt: Platelet VWF; N: Normal.

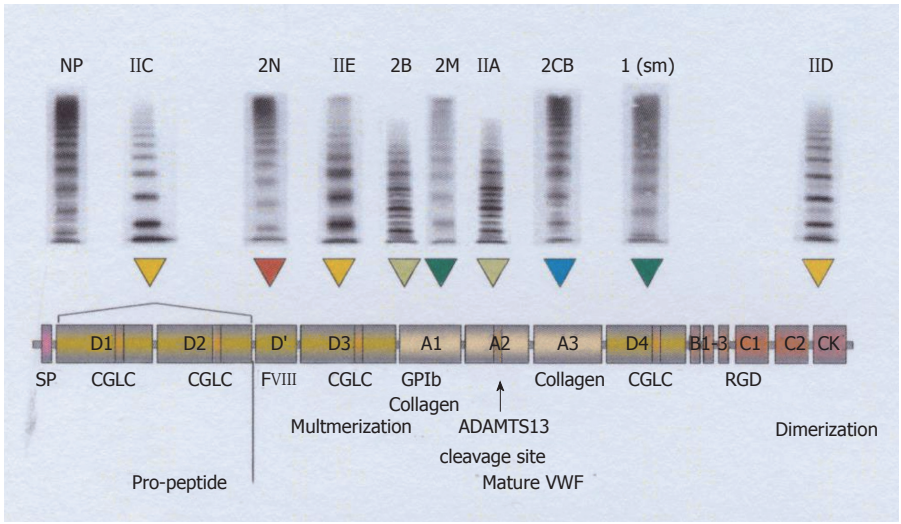

Figure 7 Translation and integration of the 2006 International Society on Thrombosis and Haemostasis and the 2009 Hamburg classification. VWD type 2 variants[20] (recessive IIC, recessive 2N, dominant IIE, 2B, 2M, IIA, 2CB 1 (sm) and IID related to clustered distribution of VWF gene mutations in the D1-D2 propeptide, D', D3, A1, A1, A2, A3, D4 and CK domains respectively. For explanation see Figure 8: from left to right recessive 2N, recessive IIC → 2C, and dominant → IIE → 2E, 2B, 2M, IIA → 2A, 2 CB (collagen binding defect), 1 smeary pattern (sm) and IID → 2D (from ref.[20]). VWD: Von Willebrand disease; VWF: Von Willebrand factor.

distinguish the different variants of pronounced type 1, 2N, 2M and 2E VWD at VWF levels around and below 0.15 U/mL[15]. The ISTH mainly used a "lumping" instead of a "splitting" approach for the classification of type 2 VWD (Table 1). The ISTH criteria lumped several variants of VWD IIA, IIC, IID, IIE together into type 2A with loss of large VWF multimers[13-15]. The loss of large multimers in VWD 2 is due to various mechanisms: increased proteolysis in dominant 2A and 2B VWD, defective multimerization of VWF in recessive 2C and dominant 2E, and defective dimerization of VWF (CK domain) in 2D VWD (Figures 7 and 8)[10-12,17-22]. Decreased RIPA due to loss of function in the interaction of platelet-GPIb-VWF is a typical feature of VWD 2M[18]. VWD 2M usually presents as pronounced type 1 VWD with normal VWF multimerization pattern[18,20]. VWD type 2M is frequently labeled by the ISTH classification as 2U, 2A-like or variant 2A with decreased RIPA and some loss of large VWF multimers[17,36]. VWD type Vicenza has "supranormal" VWF multimers and type

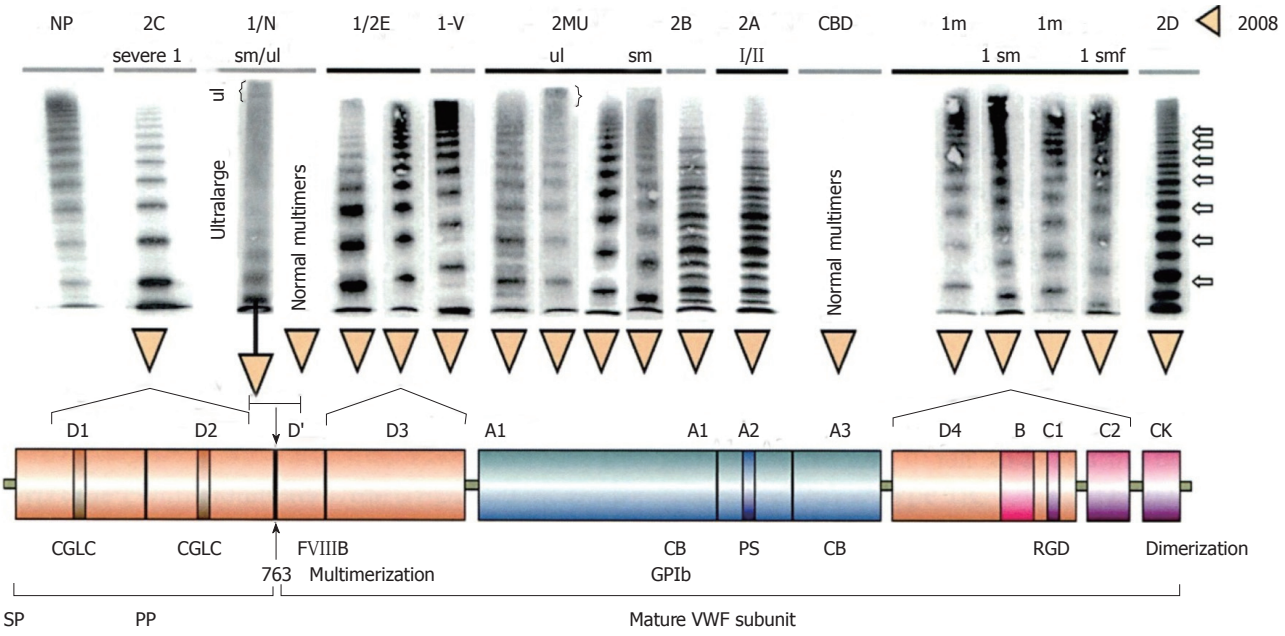

Figure 8 Structure and function of normal von Willebrand factor protein[20]. Mutations in the D1D2 domain prohibit the cleavage of VWFpp from mature VWF leading to a severe secretion and multimerization defects in recessive VWD 2C[16,31,33]. FVIII binding defects in the VWF D' domain either homozygous or double heterozygous causes recessive VWD 2N[34,35]. Dominant VWD type 2E due to heterozygous missense mutations in the D3 leads to a secretion clearance multimerization defect, VWD 2E[20,38,39]. Loss of function mutations in the VWF GpIb of the A1 domain induce dominant VWD 2M[18,36,37]. Dominant VWD 2A due to mutations in the A2 domain makes the mutant VWF hypersensitive to the VWF cleavage protease ADAMTS13 at the VWF cleavage site (1605-1606)[40-44]. Immediately after secretion the 2A mutated VWF is proteolysed with loss of large VWF multimers and typical triplet structure of each VWF band. Dominant VWD 2B due to gain of function mutation in the A1 domain accelerates the interaction of platelet-GpIb and VWF A1 followed by VWF proteolysis by ADAMTS 13 interaction[17,46]. This process starts immediately after secretion of the 2B mutated VWF and causes VWD 2B with loss of large multimers and typical triplet structure of each VWF band. A new category of VWD type 1 secretion defect (SD) is due to mutations in the D4,B1-3,C1-2[39,49] domains relabelled as the C1, C2, C3, C4, C5 and C6 domains of the VWF gene/protein[3-5]. Heterozygous mutations in the D4, C1-C6 domains result in VWD type 1 SD and have either normal multimers or abnormal multimers. Homozygous or double heterozygous mutations in the D4, C1-C6 domains are associated with severe VWD type 1[26-29]. Cysteine mutations in the CK dimerization domain, either heterozygous and homozygous or double heterozygous, are associated with VWD 2D[30]. VWD: Von Willebrand disease; VWF: Von Willebrand factor; CBD: Collagen binding defect.

Table 1 Classification of von Willebrand disease according to International Society on Thrombosis and Haemostasis guidelines 1994-2007[13-15]

1 Inherited VWD caused by genetic mutations at the VWF locus includes a broad spectrum of recessive and dominant variants of VWD
2 WD Type 1 is quantitative deficiency of VWF mainly based on a normal VWF:RCo/VWF:Ag ratio. Type 2 VWD is a qualitative deficiency of VWF as documented by a decreased VWF:RCo/VWF:Ag ratio. Type 3 refers to virtually complete deficiency of VWF
3 VWD Type 2 refers to qualitative variants with absence of high molecular weight VWF multimers and distinguishes 2A (IIA, IIB, IIE, and IID) 2B, 2M and 2 N
4 VWD Type 2M or 2U is a distinct entity with decreased platelet dependent function (VWF:RCo) and presence of large VWF multimers
5 VWD Type 2A (IIA, IIC, IIE and IID) refers to qualitative variants with absence of HMW multimers, normal or decreased RIPA and decreased VWF: VWF:RCo/VWF:Ag ratio
6 VWD Type 2B is a qualitative variant with absence of HMW multimers, decreased VWF:RCo/VWF:Ag ratio and increased RIPA
7 VWD Type 2N is a mild hemophilia due to FVIII binding defect of VWF, presence of large VWF multimers, normal VWF:RCo/VWF:Ag ratio and decreased FVIII/VWF:Ag ratio

VWD: Von Willebrand disease; VWF: Von Willebrand factor; RIPA: Ristocetin-induced platelet aggregation.

1 phenotype due to increased clearance[18,32]. In type Vicenza the multimers are cleared too rapidly for ADAMTS13 mediated proteolysis to occur. In the ISTH classification, VWD 2N has normal VWF multimers, a typical type 1 VWD phenotype with low FVIII:C and decreased FVIII:C/VWF:Ag ratio[13-15]. Between 2001 and 2009 Schneppenheim *et al*[10,11,20], and Michiels *et al*[17] translated and modified the ISTH classification of VWD type IIA, IIB, IIC and IIE (Table 1) into the European Clinical, Laboratory and Molecular (ECLM) criteria (Table 2) for VWD recessive 2C, recessive 2N, dominant 2E, 2M, 2A, 2B, 2CBD and 2D (Figures 6, 7 and 8)[32,33,37-39].

The distinction of the dominant type 2 VWDs in the ECLM classification is based on typical VWF multimeric patterns for each type 2 VWD variant in high resolution gel concentration (1.5%)[10,11,20].

Pronounced dominant type 1 VWD with VWF levels around and below 0.15 U/L using the ISTH criteria is seen in VWD type 1 secretion or clearance defects, and in VWD type 2E and 2M (Figure 8)[17,18,20]. Diagnostic differentiation of so-called severe type 1 VWD using the ISTH criteria remains a persistent problem in routine daily practice anno 2011 (Table 1). This can easily be overcome by the use and correct interpretation of

Table 2 European Clinical, Laboratory and Molecular criteria of von Willebrand disease

Mild type 1: VWF:Ag < 35%, normal VWF:CB/VWF:Ag and VWF:RCo/VWF:Ag ratio > 0.7
Type 1 with VWF:Ag above 35% with manifest bleeding can be included
Autosomal recessive VWD
Type 3 recessive with VWF:Ag and FVIII:C undetectable
Type 1 severe recessive VWD with VWF:Ag and VWF:RCo detectable < 5%, high FVIII:C/VWF:Ag ratio in particular after DDAVP
Type 2C recessive with increased FVIII:C/VWF:Ag ratio (secretion defect) and loss of large VWF mutimers due a mulimerization defect caused by homozygous or double heterozygous mutations in the D1-D2 of the VWF gene (Figure 8)
Type 2N recessive with FVIII:C/VWF:Ag ratio < 0.5 due to FVIII-VWF binding defect caused by mutations in the D' FVIII-binding domain (Figure 8)
Type 2 autosomal dominant VWD 2A, 2B, 2E and 2M (Figure 8)
2A/2M: Decreased RIPA (Ristocetin Induced Platelet Aggregometry, 2B increased RIPA, decreased VWF:RCo/VWF:Ag ratio < 0.7
2A: Loss of large MM caused by increased VWF proteolysis due to mutations in the A2 domain of the *VWF* gene
2B: Increased RIPA (0.8 mg/mL) and thrombocytopenia with VWD type 2 due to gain of function mutation in the GpIb receptor in the A1 domain
2E: Type 1/2, loss of large multimers due to multimerization defect and increased clearance due to mutations in the D3 multimerization domain
2M: Decreased VWF:RCo/VWF:Ag ratio (< 0.6), normal VWF:CB/VWF:Ag ratio (> 0.7), decreased RIPA due to loss of function mutation in the A1 domain
2M-CBD: Collagen binding defect, VWF:RCo/VWF:Ag ratio > 0.7 and VWF:CB/VWF:Ag ratio < 0.7 due to mutation in the A3 domain

VWD: Von Willebrand disease; VWF: Von Willebrand factor; **RIPA: Ristocetine induced platelet aggregation; MM: Multimers.**

Table 3 Desmopressin challenge test (0.3 μg/kg in 100 mL physiological saline intravenously over 30 min) proposed by the International Society on Thrombosis and Haemostasis

Blood sample DDAVP	At 15 min	After DDAVP				After DDAVP 12 h
		1 h	2 h	4 h	6 h	
Ivy BT	+	-	+	-	-	+
PFA-100	+	+	+	+	+	+
RIPA	+	+	+	+	+	+
FVIII:C	+	+	+	+	+	+
VWF:Ag	+	+	+	+	+	+
VWF:RCo	+	+	+	+	+	+
VWF:CB	+	+	+	+	+	+
VWF:MM	+	+	+	+	+	+
VWF propeptide	+	+	+	+	+	+

This challenge test has been used at the Goodheart Institute, Rotterdam since 1992 to calculate the recovery and half life times of FVIII:C and VWF parameters for the diagnosis and charaterization of VWD type 1, 2 and 3[18]. VWD: Von Willebrand disease; VWF: Von Willebrand factor; **RIPA: Ristocetin induced platelet aggregation; DDAVP: Desmopressin; MM: Multimers.**

VWF multimeric analysis and FVIII:C/VWF:Ag response curves to DDAVP[18-20]. VWF multimeric analysis using low and medium resolution gels clearly distinguishes VWD type 2A, 2B, 2E and 2M (Figure 8, middle part)[32]. The responses of FVIII and VWF parameters to intravenous DDAVP is an essential tool in the splitting approach of the ECLM classification; it allows to distinguish the various variants of dominant type 1 and 2, and elucidates the molecular differences between homozygous or compound heterozygous recessive type 3 and severe type 1 VWD[16,33]. The ECLM splitting approach uses sensitive and specific diagnostic tools with regard to structure and function defects of mutant VWF proteins (Table 2).

Characteristics of dominant type 1 VWD secretion defect, 2M and 2E

FVIII:C and VWF parameters in dominant VWD type 1 secretion defect are characterized by increased FVIII:C/VWF:Ag ratio before and after DDAVP with restricted responses of the VWF parameters as compared to FVIII response to DDAVP (Figure 9)[18]. We studied three family index cases with pronounced autosomal dominant cases of VWD type 1, in whom the responses to DDAVP of all VWF parameters were very restrictive, whereas FVIII:C levels reached very high levels around 2.0 U/mL. This discrepancy of increased FVIII:C/VWF:Ag ratio and restricted responses to DDAVP of all VWF parameters is diagnostic for a pronounced VWD type 1 secretion defect[18,19] and is clearly different from VWD 2M (Figure 9)[18,20]. VWD 2M has normal VWF multimers before and after DDAVP (Figures 9 and 10) and the responses to DDAVP are poor for VWF:RCo, fairly good for VWF:CB, FVIII:C and VWF:Ag, followed by shortened half-life times of FVIII:C, VWF:Ag and VWF:CB, indicative for a clearance defect (Figures 9 and 10)[18,20,36,37].

The response to DDAVP in a case of dominant VWD type 2E due to *W1120S* mutation in the A3 domain induced transient correction of PFA-100 closure time and restricted increase of VWF parameters from around 0.20-0.40 U/mL to around 1.0 U/mL (Figure 10). The VWD type 2E usually presents as laboratory phenotype 1 or 2, but the the multimeric pattern is charaterized by loss of large multimers and the absence of the triplet structure of VWF bands due to mutations in the D3 multimerization domain (Figure 8)[38,39].

Dominant VWD type 2A Group I and II

The missense mutations *V1607D*, *S1506L*, *L1540P* and *R1568del* result in poor or no secretion of high molecular weight multimers due to intracellular proteolysis and impaired transport of VWF multimers between the endoplasmatic reticulum and the Golgi complex (so-called VWD 2A Group 1 defect)[40-44]. Eight missense mutations in the A2 domain (*R1597W*, *G1505E*, *I1628T*, *L1503Q*, *M1528V*, *G1609R*, *I1628T*, *G1629E*, *G1631D* and *E1638K*) result in normal secretion of high molecular weight multimers, which are hypersensitive to ADAMTS13-induced proteolysis

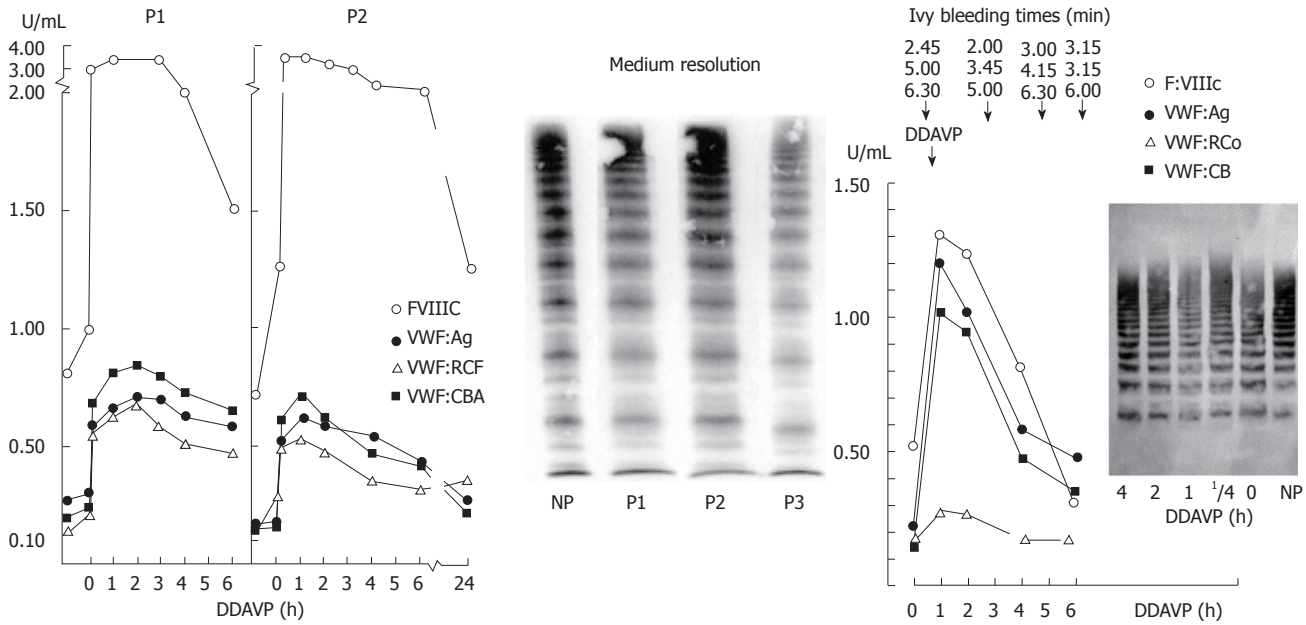

Figure 9 Restricted response of von Willebrand factor parameters to desmopressin. Pronounced dominant VWD type 1 secretion defect (high FVIII:C/VWF:Ag ratio) with restricted response of VWF parameters to DDAVP as compared to completely normal responses of FVIII (high FVIII:C/VWF:Ag ratio) is indicative for VWD type 1 secretion defect (Left). Diagnostic differentiation of pronounced 1 VWD 1 secretion defect with normal VWF multimers (VWF MM according to Budde) and restricted decreased response to DDAVP of all VWF parameters in two members of one family (proband and her brother) *vs* pronounced case of dominant VWD 2M (Right) with normal VWF multimers before and after DDAVP[18], a poor response of VWF:RCo to DDAVP and fairly good responses to DDAVP of FVIII:C, VWF:Ag and VWF:CB followed by shortened half life times of FVIII:C, VWF:Ag and VWF:CB indicative for rapid clearance defect. Dominant VWD type 2M (Michiels) is featured by loss of function mutation in the A1 domain, normal multimers, decreased to zero RIPA, low VWF:RCo activity, a secretion defect and rapid clearance[18]. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; NP: Normal plasma; P: Patient.

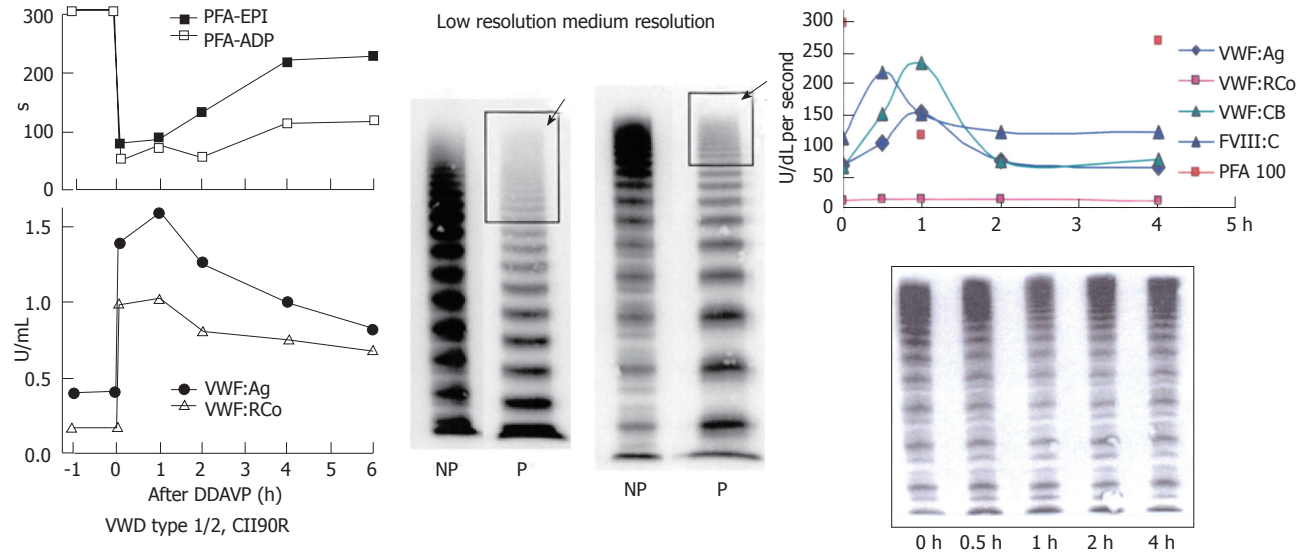

Figure 10 Von Willebrand disease 2E (left) and von Willebrand disease 2M (right). Left: Dominant VWD type 2E: multimerization defect with loss of large VWF multimers to W1120S mutation in the A3 domain. DDAVP induced transient correction of PFA-100 closure time and restricted increase of VWF parameters from around 0.20-0.40 U/mL to around 1.0 U/mL. In VWD type 2E, VWF multimeric pattern is characterized by a lack or relative decrease of large multimers and the absence of the outer sub-band of the normal triplet structure. Medium resolution gel according to Budde *et al*[12]. Right: VWD 2M: Poor response of VWF:RCo to DDAVP, normal VWF multimers before and after DDAVP and good responses of FVIII, VWF:Ag and VWF:CB followed by shortened half-life time indicating rapid clearance defect of the FVIII-VWF complex on top of loss of VWF:RCo function in VWD 2M[20]. Medium resolution gel according to Budde *et al*[12]. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; PFA-EPI: Platelet function analyzer, epinephrin; PFA-ADP: Platelet function analyser adenosine di-phosphate; NP: Normal plasma; P: Patient.

(so-called VWD 2A Group 2 defect)[40-43]. VWF of severe VWD 2A Group I is already proteolysed in endothelial cells before secretion, whereas VWF in mild to moderate VWD 2A Group II is secreted as large multimers, which after secretion from endothelial cells are proteolysed due to hypersensitivity to ADAMTS13[40-44].

Dominant VWD type 2A mutation V1499E in a large Dutch family is featured by normal RIPA, loss of large

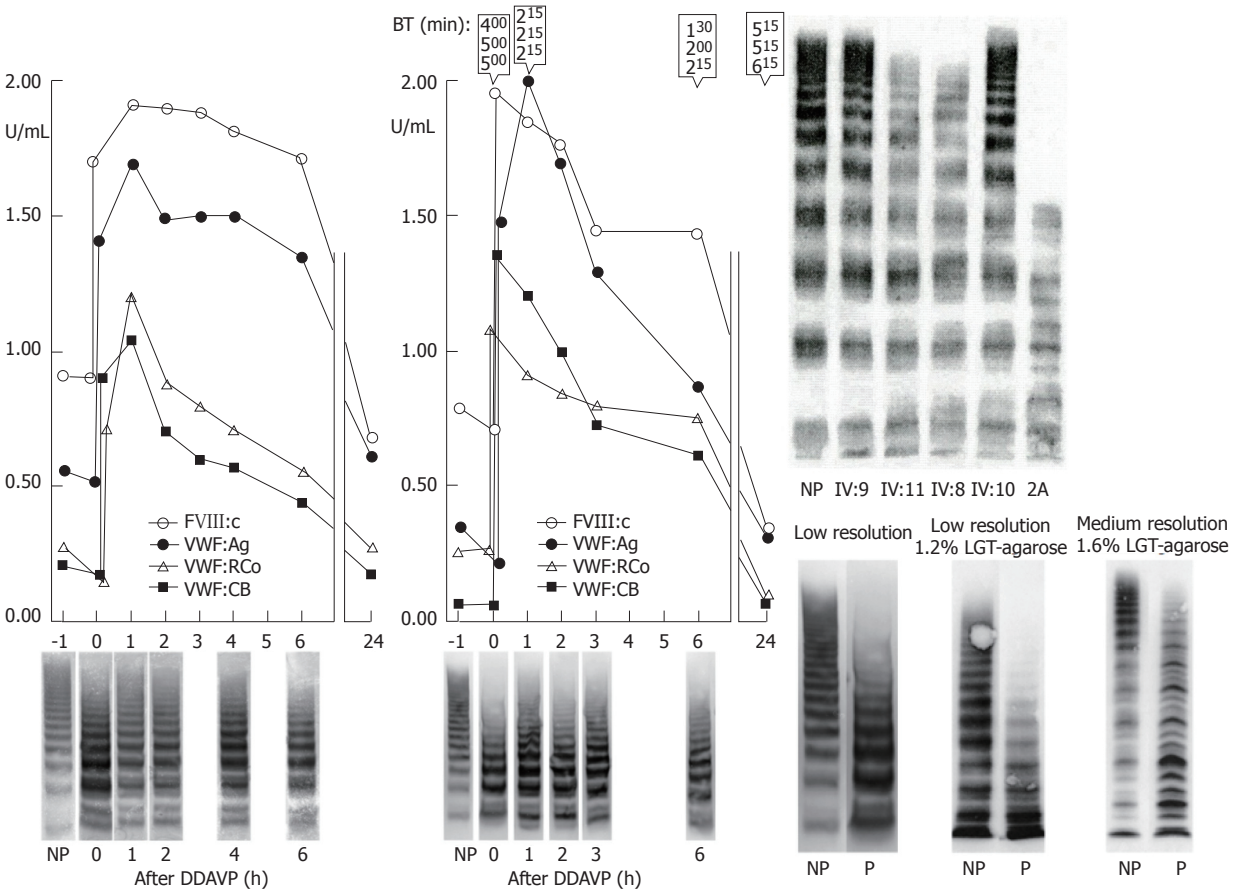

Figure 11 Dominant von Willebrand disease type 2A mutation *V1499E* is featured by a normal ristocetin-induced platelet aggregation assay. The loss of largest VWF multimers and increase of intermediate and small VWF multimers in low resolution gels (VWF multimeric pattern before DDAVP, lower left)[43,44]. The responses to DDAVP of FVIII:C and von Willebrand factor antigen (VWF:Ag) are normal. The responses to DDAVP of the functional VWF:RCF and VWF:CB are restricted to about 1 U/mL 1 h post-DDAVP with transient correction of Ivy bleeding times and transient reappearance of large VWF multimers in two cases of moderate dominant VWD type 2A (mutation *V1499E*). As compared to VWF:Ag and FVIII:C, the half life times of VWF:RCo and VWF:CB are shortened due to increased proteolysis of VWF multimers (Left). Lower right: Please note that the VWF multimers in low resolution gels in the Rotterdam laboratory and in the Hamburg Laboratory (Budde, middle lanes) clearly show the absence of large VWF multimers and no triplet of the individual VWF bands. The typical triplet structure of the individual VWF bands diagnostic for VWD type 2A was only seen in the medium resolution gels (right lanes) according to Budde. Upper right: The multimeric analysis of VWF from affected patients from the large Dutch family with dominant V1499E mutated VWD 2A in a third laboratory (Amsterdam)[43] show the loss of the largest VWF multimers as shown for 2 affected cases (IV:8 and IV:11) as compared to normal (NP) and 2 non-affected family members (IV:9 and IV:10). The loss of large multimers in *V1499E* mutated VWD patients was less pronounced as compared to a case of typical VWD 2A with the loss of large and some of the intermediate VWF multimers and a typical triplet structure of each VWF band in that laboratory[43]. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; NP: Normal plasma; P: Patient.

VWF multimers and increase of intermediate and small VWF multimers in low resolution gels (VWF multimeric pattern before DDAVP, Figure 11, lower left)[43,44]. The responses to DDAVP are normal for FVIII:C but restricted for the functional VWF:RCo and VWF:CB to about 1 U/mL 1 h post-DDAVP. Transient correction of Ivy bleeding times was associated with temporary reappearance of large VWF multimers indicating that the mutation *V1499E* belongs to VWD 2A Group II (Figure 11). The multimeric pattern of the V1499E mutant VWF was studied in three different laboratories. Low resolution gels in two laboratories clearly show the absence of large VWF multimers but no clear triplets of individual VWF bands (Figure 11). The triplet structure of the individual VWF bands diagnostic for VWD type 2A was only seen in the medium resolution gels (right lanes, Figure 11). Severe VWD 2A Group I is characterized by pronounced triplet structure, absence of RIPA and prolonged Ivy bleeding times as shown in our case with the S1506L mutation in the A2 domain (Figure 12). The poor responses to DDAVP of the VWF parameters are completely in line with impaired assembling, transport and proteolysis of intracellular VWF multimers seen in severe VWD 2A Group I caused by mutations like S1506L (Figure 12).

Dominant VWD type 2B

The key feature of VWD 2B is the loss of large VWF multimers (Figure 8, Table 2) due to increased protelolysis caused by increased interaction of platelets and mutated VWF in the A1 domain (increased RIPA)[18,20,45,46]. The process of increased VWF-GpIb-platelet interaction of mutant VWF in VWD 2B starts as soon as the mutant VWF enters the circulation (Figure 4).

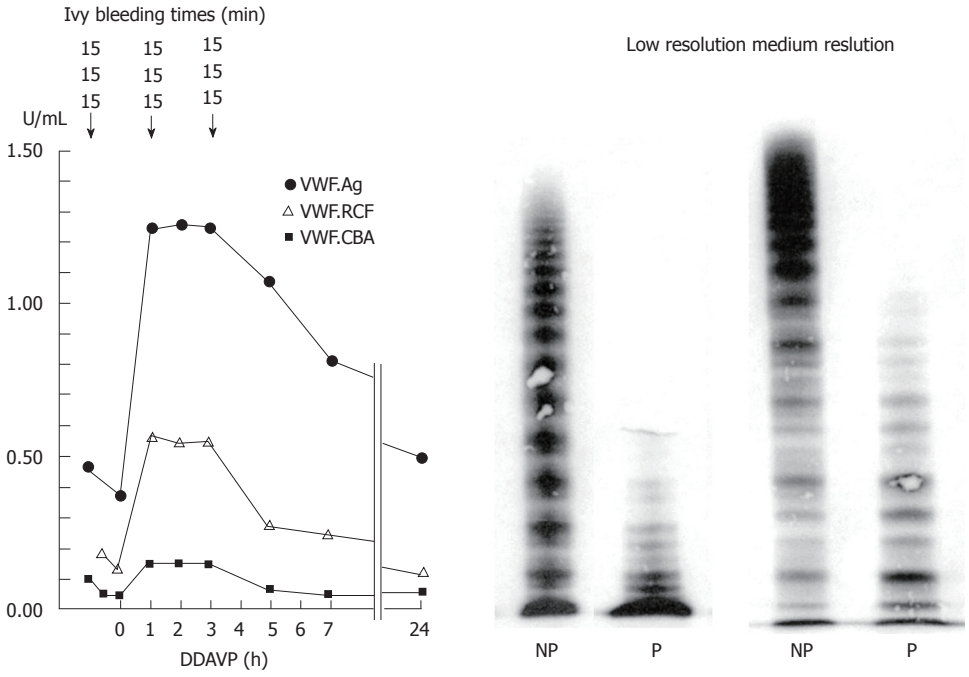

Figure 12 Absence of large and intermediate von Willebrand factor multimers in severe dominant von Willebrand disease type 2 A, with absence of ristocetin-induced platelet aggregation and strongly prolonged Ivy bleeding times in a case with the *S1506L* mutation[44]. The responses of VWF parameters to DDAVP are very poor with no correction of Ivy bleeding times (BT) and no re-appearance of large VWF multimers in this case with dominant severe VWD 2A Group I indicating impaired assembling, transport and proteolysis of intracellar VWF multimers caused by the mutation *S1506L* near to the VWF cleavage site in the A2 domain of the *VWF* gene. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; NP: Normal plasma; P: Patient.

Clumps of mutant VWF-platelets are cleared from the circulation leading to thrombocytopenia upon DDAVP or stress. Federici *et al*[46] evaluated the clinical and molecular predictors of thrombocytopenia and the risk of bleeding in 67 VWD 2B patients from 38 unrelated families. Thrombocytopenia was found in 30% at baseline and in 57% after stress conditions in only those with pronounced VWD 2B carrying the mutation[46]. Thrombocytopenia did not occur in 16 patients (24%) from 5 families with mild VWD 2B carrying the *P1266L* or *R1308L* mutation[46]. The *P1288L* and *R1308L* mutations are associated with a mild type 1 variant of VWD 2B with normal VWF:RCo/VWF:Ag ratios of 0.9 and 0.8 respectively, also seen in P1266L-mutated VWF in VWD Malmo and New York VWD phenotype 1B, who do have a mild bleeding illness with normal VWF:RCo/VWF:Ag ratios consistent with a laboratory VWD type 1B phenotype with increased RIPA[17,18].

MYSTIFICATIONS AROUND ISTH-DEFINED VWD TYPE 1

The European (EU) study on ISTH-defined type 1 VWD, named EU MCMDM-1VWD[44,45], involved twelve partners in nine European countries, and aimed to recruit the whole spectrum of patients diagnosed by referring centres as having type 1 VWD, including the more severe and mildest cases, to try and represent the range of patients seen by other centers diagnosing type 1 VWD. The EU MCMDM-1VWD study recruited 148 evaluable families. The Canadian type 1 VWD study recruited 124 families from 13 Haemophilia Centres across Canada[47-49]. Analysis at both the recruitment centre and central laboratory of plasma samples was obtained on at least two occasions. The EU and Canadian VWD 1 multi-centre national/international studies have provided new insights into the molecular pathogenesis of type 1 VWD. In 2008, 117 different VWF mutations (80% missense, about 10% non-sense and about 10% splice site or transcription) were reported to be associated with type 1 VWD and were included in the ISTH VWF mutation database. When comparing the ECLM criteria in Table 2 with the ISTH criteria in Tables 1 and 3, there are several misclassifications of VWD in the European MCMDM-1VWD study. The European MCMDM-1VWD study did contain typical examples of recessive or heterozygous VWD type 2N (heterozygous R816W, R854W and R854W/R924Q, R854W/null) and typical cases of VWD 2M (D1277-E78delinsl, R1315C, R1342CR1374C, R1374H, G1415D I1416N)[44]. There were 3 cases with typical 2M VWD with abnormal multimers and 2 mutations (R1315H/P1266L, R1315L/R934Q and R1374C/P2145S) in which the 2M mutation has a dominant negative effect on the VWD type 1 mutation[47]. The mutations in exon 26, D3 domain, R1130R/G/F, W1144G, Y1146C and C1190R usually present with a laboratory phenotype VWD 1 but have abnormal VWF multimers with typical features of VWD 2E[39,49]. The majority of mild type 1 VWD cases in the Canadian study were in fact carriers of recessive severe type 1 VWD heterozygous for mutations mainly

located in the D1-D2 and D'domains (K762E, M771I, P812fs, Exon 21 skip, R924Q, R924W and C996E)[50,51]. A minority of ISTH-defined type 1 VWD patients in the Canadian study had missense mutations in the D3 (S1024fs, I1094T, in fact VWD 1/2E), A1 (F1280fs, R1379C, P1413L, Q1475X, in fact VWD 2M) or A2 domain (R1583W, and Y1584C)[52-54]. The combination of C1584/bloodgroup O is rather frequent and typically shows a good to normal response to DDAVP[53,54]. Carriers of recessive VWD type 3 or severe recessive type 1 VWD are asymptomatic or may manifest mild bleeding in particular when associated with blood group O[16,33].

CONCLUSION

The classification of VWD remains an important problem to this day. Several classifications have been proposed but none have proved to be ideal. The current ISTH classification is a lumping together of types based upon easily available but "insensitive" laboratory techniques, with especially type 2A as a collection of different pathophysiological entities. The ECLM criteria for VWD try to improve on this classification by including also the response to DDAVP, and have more regard to pathophysiology and the VWF domain structure.

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ORIGINAL ARTICLE

Basic Study

Identifying changes in punitive transcriptional factor binding sites from regulatory single nucleotide polymorphisms that are significantly associated with disease or sickness

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Abstract

AIM

To identify punitive transcriptional factor binding sites (TFBS) from regulatory single nucleotide polymorphisms (rSNPs) that are significantly associated with disease.

METHODS

The genome-wide association studies have provided us with nearly 6500 disease or trait-predisposing SNPs where 93% are located within non-coding regions such as gene regulatory or intergenic areas of the genome. In the regulatory region of a gene, a SNP can change the DNA sequence of a transcriptional factor (TF) motif and in turn may affect the process of gene regulation. SNP changes that affect gene expression and impact gene regulatory sequences such as promoters, enhancers, and silencers are known as rSNPs. Computational tools can be used to identify unique punitive TFBS created by rSNPs that are associated with disease or sickness. Computational analysis was used to identify punitive TFBS generated by the alleles of these rSNPs.

RESULTS

rSNPs within nine genes that have been significantly associated with disease or sickness were used to illustrate the tremendous diversity of punitive unique TFBS that can be generated by their alleles. The genes studied are the adrenergic, beta, receptor kinase 1, the v-akt murine thymoma viral oncogene homolog 3, the activating transcription factor 3, the type 2 demodkinase gene, the endothetal Per-Arnt-Sim domain protein 1, the lysosomal acid lipase A, the signal Transducer and Activator of Transcription 4, the thromboxane A2 receptor and the vascular endothelial growth factor A. From this sampling of SNPs among the nine genes, there are 73 potential unique TFBS generated by the common alleles compared

to 124 generated by the minor alleles indicating the tremendous diversity of potential TFs that are capable of regulating these genes.

CONCLUSION

From the diversity of unique punitive binding sites for TFs, it was found that some TFs play a role in the disease or sickness being studied.

Key words: Regulatory single nucleotide polymorphisms; Alleles; Transcriptional factors; Transcriptional factor binding sites; Linkage disequilibrium; Disease or sickness

Core tip: Disease or trait-predisposing single nucleotide polymorphisms (SNPs) in or near genes can alter the transcriptional factor binding sites (TFBS) for the TFs regulating the gene; thereby affecting the health of an individual. In this report, the disease or sickness associated regulatory SNPs (rSNPs) within a sampling of nine human genes were studied with respect to the alterations in TFBS. From this sampling there were 73 punitive unique TFBS generated by the common rSNP alleles compared to 124 generated by the minor alleles indicating the tremendous diversity of potential TFs that are capable of affecting the health of person.

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INTRODUCTION

The genome-wide association studies (GWAS) have over the past decade provided us with nearly 6500 disease or trait-predisposing single nucleotide polymorphisms (SNPs). Only seven percent of these SNPs are located in protein-coding regions of the genome[1,2] while the remaining 93% are located within non-coding regions[3,4] such as gene regulatory or intergenic areas of the genome. Much attention has been drawn to SNPs that occur in the putative regulatory region of a gene where a single nucleotide change in the DNA sequence of a potential transcriptional factor (TF) motif may affect the process of gene regulation[5-7]. A nucleotide change in a transcriptional factor binding site (TFBS) can have multiple consequences. Since a TF can usually recognize a number of different binding motifs in a gene, the SNP may not change the TFBS interaction with the TF and consequently not alter the process of gene expression. In other cases the nucleotide change may increase or decrease the TF's ability to bind DNA which would result in allele-specific gene expression. In some cases a nucleotide change may eliminate the natural binding motif or generate a new binding site (BS) as a result the gene is no longer regulated by the original TF[8,9]. Single nucleotide changes that affect gene expression by impacting gene regulatory sequences such as promoters, enhancers, and silencers are known as regulatory SNPs (rSNPs)[5,6,10,11]. Therefore, functional rSNPs in TFBS may result in differences in gene expression, phenotypes and susceptibility to environmental exposure[7]. Examples of rSNPs associated with disease susceptibility are numerous and several reviews have been published[7,12-16]. Advances in understanding the functional relevance of SNPs in non-coding regions of the human genome using epigenomics and genome engineering have been recently reviewed[17]. Computational tools can be used to identify punitive TFBS created by rSNPs which are associated with disease or sickness[18]. To this end, computational analysis has been used to identify punitive or potentially unique TFBS generated by the alleles of rSNPs[19] where unique TFBS occur with only one of the two rSNP alleles.

In this report, rSNPs within a sample of nine human genes (Table 1) which have been significantly associated with disease or sickness were selected to illustrate the tremendous diversity of unique punitive TFBS that can be generated by SNP alleles (Table 2)[8,9,20-27]. The SNP alleles from these reports were found to share common TFBS between alleles but each SNP allele can also create unique TFBS only for that allele (Table 2). As an example in Table 2, the rs948988 ADRBK1-G allele creates two potential unique TFBS for the Kruppel-like factors 1 and 4 (KLF1,4) TFs that do not occur with the alternate ADRBK1-A allele while the ADRBK1-A allele creates ten other punitive unique TFBS not found with ADRBK1-G allele. Many of the rSNPs have been reported to be in linkage disequilibrium (LD) (Table 1), where LD is considered to be the non-random association of SNP alleles within a gene. LD between SNPs in the regulatory region of a gene can indicate strong associations of certain haplotypes and TFBS with sickness or disease[28].

MATERIALS AND METHODS

Identifying TFBS

The JASPAR CORE database[29,30] and ConSite[31] were used to identify the TFBS in this study. JASPAR is a collection of transcription factor DNA-binding preferences used for scanning genomic sequences where ConSite is a web-based tool for finding cis-regulatory elements in genomic sequences. The Vector NTI Advance 11 computer program (Invitrogen, Life Technologies) was used to locate SNPs and TFBS within all genes listed in Table 1.

RESULTS

The protein and gene symbol, chromosome position of the gene, SNP number and location within the gene and nucleotide (mutation) change are listed in Table 1. Also listed is whether or not linkage disequilibrium occurs

Table 1 Genes and their single nucleotide polymorphisms that have been found to be associated with disease or sickness

Protein and gene symbol	Chromosome	SNP	SNP location	Mutation	LD	Ref.
Adrenergic, beta, receptor kinase 1	11q13.1	rs948988	intron 2	c.190 + 653G > A	Yes	[9,19]
		rs4370946	3'UTR	c.*217C > T	Yes	
v-akt murine thymoma viral oncogene homolog 3	1q44	rs4590656	intron 1	c.46 + 3654C > T	Yes	[8,19]
		rs10157763	intron 1	c.46 + 11386C > T	Yes	
		rs2125230	intron 1	c.47-26830G > A	Yes	
Activating transcription factor 3	1q32.3	rs3125289	promoter	c.-5 + 9322T > C	Unknown	[19,20]
		rs11119982	promoter	c.-4-23516C > T	Unknown	
Type 2 demodkinase gene	14q24.3	rs225015	3'UTR	c.*1453G > A	Yes	[19,21]
		rs225011	intron 1	c. 330 + 366C > T	Yes	
		rs12885300	5'UTR	c.-451C > T	Yes	
Endothetal Per-Arnt-Sim domain protein 1	2p21	rs6756667	intron 2	c.218-3881A > G	No	[22]
		rs1868092	3'UTR	c.*2403G > A	No	
Lysosomal acid lipase A	10q23.31	rs1412444	intron 2	c.229 + 2506C > T	n/a	[23]
Signal transducer and activator of transcription 4	2q32.3	rs8179673	intron 2	c.274-28290T > C	Yes	[24]
		rs10181656	intron 2	c.274-28828C > G	Yes	
Thromboxane A2 receptor	19p13.3	rs2238631	intron 1	c.-84 + 2229G > A	Yes	[19,25]
		rs2238632	intron 1	c.-84 + 2030C > T	Yes	
		rs2238634	intron 1	c.-84 + 1799G > T	Yes	
Vascular endothelial growth factor A	6p21.1	rs34357231	promoter	c. -2550-2568D > I	Yes	[19,26,28]
		rs1570360	promoter	c.-614A > G	Yes	
		rs3025039	3'UTR	c.*237C > T	Yes	

Also listed is the gene chromosome location, single nucleotide polymorphism location in the gene and the resulting genetic mutation as well as the occurrence of linkage disequilibrium between single nucleotide polymorphisms found within each gene. SNP: Single nucleotide polymorphism; LD: Linkage disequilibrium; n/a: Not available.

Table 2 Genes whose single nucleotide polymorphisms are significantly associated with human disease or sickness

Gene symbol							
ADRBK1	Ethnic group	B		B			
	disease or sickness	12		12			
	SNP	rs948988 (G/A)		rs4370946 (C/T)			
	alleles (MAF)	G	A (0.29)	C	T (0.2)		
	potential unique TFBS	KLF1, 4	BATF:JUN	E2F1,3,4,6	ARNT:AHR		
			ESR2	EGR1	ATOH1		
			FOS	INSM1	ELF1		
			FOSL2	KLF4	ESR2		
			JUND	NFKB1	NR3C1		
			JUN:FOS	NRF1			
			MYB	SP1, 2			
			NFE2L1:MAF				
			NR3C1				
			SOX17				
AKT3	Ethnic group	G		C		C	
	disease or sickness	1		14		14	
	SNP	rs4590656 (C/T)		rs10157763 (C/T)		rs2125230 (G/A)	
	alleles (MAF)	C	T (0.41)	C	T (0.33)	G	A (0.2)
	potential unique TFBS	ARNT:AHR	GFI	ELF5	CTCF	ARNT:AHR	GATA1
		HIF1a:ARNT	HNF4A	ELK1	NFATC2	FEV	HNF4a
			PAX2	MYCN	SOX17	HIF1a:ARNT	HOXA5
			SPIB	SPIB	ZNF354C	SPI1	IRF1
				SPI1			NR2F1
				TFAP2A			SOX17
ATF3	Ethnic group	C		C			
	disease or sickness	15		15			
	SNP	rs3125289 (C/T)		rs11119982 (C/T)			
	alleles (MAF)	C	T (0.10)	C	T (0.36)		
	potential unique TFBS	ARNT	FOXA1, 2	HLTF	ARID3A		
		ARNT:AHR	FOXL1		MAX		
		GABPa	FOXO3		MYB		
		MYC	HLTF		USF1		
		MYCN	SOX10		ZEB1		
		MZF1	SOX17				
		SPIB	SRY				
		USF1					

DIO2	Ethnic group	F		F		C	
	disease or sickness	2		2		17	
	SNP	rs225015 (G/A)		rs225011 (C/T)		rs12885300 (C/T)	
	alleles (MAF)	G	A (0.4)	C	T (0.42)	C	T (0.23)
	potential unique TFBS	EBF1	ELF1	CRX	FOXL1		ARID3A
		ESRRA	ELK1	RXRa	MEF2A		BATF:JUN
		PPARg:RXRa	ERG		PDX1		IRF1
		RFX5	ETS1				JUN:FOS
		THAP1	FLI1				PAX2
			RUNX1				SOX6
			SOX9				
			SPI1				
			TCF7L2				
EPAS1	Ethnic group	G		G			
	disease or sickness	1		1			
	SNP	rs6756667 (A/G)		rs1868093 (A/G)			
	alleles (MAF)	A	G (0.20)	A	G (0.25)		
	potential unique TFBS	CEBPa	ATF7	NR2C2	HIC2		
		NFIA	GMEB2	NFIA	KLF5		
		NRL	JDP2	YY1	MGA		
					TEAD1		
					USF1		
LIPA	Ethnic group	C, D, E					
	disease or sickness	20, 21					
	SNP	rs1412444 (C/T)					
	alleles (MAF)	C	T (0.32)				
	potential unique TFBS	ELF1	FOXA1				
		ETS1	FOXL1				
		GABPa	FOXO3				
		HOXA5	HNF1B				
		SPI1	MEF2A				
			NFKB1				
			NFIC				
			PAX2				
			SOX6				
			SOX9				
			SRY				
			THAP1				
STAT4	Ethnic group	A, C		A, C			
	disease or sickness	2,6,10		2,10			
	SNP	rs8179673 (T/C)		rs10181656 (C/G)			
	alleles (MAF)	T	C (0.26)	C	G (0.26)		
	potential unique TFBS	EN1	FOXA2	AR	HNF4g		
		NFIL3	FOXH1	E2F6	STAT3		
			FOXO1	NR1H3:RXRa			
			FOXP1	ZNF263			
			FOXQ1				
			HNF1a				
			HNF4g				
TBXA2R	Ethnic group	A		A		A	
	disease or sickness	22		22		22	
	SNP	rs2238631 (G/A)		rs2238632 (C/T)		rs2238634 (G/T)	
	alleles (MAF)	G	A (0.2)	C	T (0.21)	G	T (0.22)
	potential unique TFBS	FOXC1	ELK1		ARNT		HLTF
		TFAP2a	ELK4		CREB1		HNF4a
			ETS1		HIF1a:ARNT		NR2F1
			GATA2		MAX		NR2E3
			HAND1:		USF1		NR4A2
			TCFE2a				
			SPZ1				
VEGFA	Ethnic group	G		G		G	
	disease or sickness	1		1		1	
	SNP	rs34357231 (I/D)		rs1570360 (G/A)		rs3025039 (C/T)	
	alleles (MAF)	D	I (0.28)	G	A (0.13)	C	T (0.09)
	potential unique TFBS	HNF4a	AR	EGR1	EGR2	BRCA1	NFE2::MAF
		HNF4g	EGR1,2	MZF1	EHF	ESR2	RFX5
		JUN	KLF5	SP2	FOXH1	HIF1A:ARNT	YY1

		MYB	MZF1_1-4	MAFK	NFE2L1:MAFG
		NFIC	NFYB	SPIB	
		NR2C2	NFATC2	THAP1	
		NR4A2	NKX2-5		
		PAX2	NKX3-2		
		RFX5	SP1, 2		
			STAT5A:		
			STAT5B		
Ethnic Group	Disease		Disease		Disease
A. Asian	1. Chronic mountain sickness		8. Juvenile idiopathic arthritis		15. Hypospadias
B. Black	2. Diabetes		9. Primary biliary cirrhosis and Crohn's disease		16. Mental retardation
C. Caucasian	3. Hepatitis B virus- related hepatocellular		10. Lupus		17. Osteoarthritis
D. Chinese	4. Hepatitis B virus infection		11. Ulcerative colitis		18. Insulin resistance
E. Hispanic	5. HBV viral clearance		12. Cardiovascular disease		19. Hepatic glucose output
F. Pima Indians	6. Hepatocellular carcinoma		13. Renal cell carcinoma risk		20. Coronary artery disease
G. Tibetan	7. Inflammatory bowel disease		14. Aggressive prostate cancer		21. Myocardial infarction
					22. Asthma

Also listed are the SNP alleles and frequencies within the ethnic group as well as the potential unique transcriptional factor binding site created with each SNP allele. For a complete list of significant gene SNPs see references (Table 1). SNP: Single nucleotide polymorphisms; ADRBK1: Adrenergic, beta, receptor kinase 1; AKT3: V-akt murine thymoma viral oncogene homolog 3; ATF3: Activating transcription factor 3; DIO2: Type 2 demodkinase gene; EPAS1: Endothetal Per-Arnt-Sim domain protein 1; STAT4: Signal transducer and activator of transcription 4; TBXA2R: Thromboxane A2 receptor; VEGFA: Vascular endothelial growth factor A; LIPA: Lysosomal acid lipase A.

between the SNPs within each gene (Table 1). Nine genes, ethnic groups, disease or sickness, SNPs and alleles as well as potential unique TFBS per allele that have been reported are found in Table 2. Not all of the SNPs for each gene are listed in the tables but can be found in the accompanying references (Table 1). From Table 2, it can be seen that there occur incidences when the SNP common allele does not have any unique punitive TFBS while the minor allele provide several (*e.g.,* rs12885300 in DIO2; rs2238632 and rs2238634 in TBXA2R). There are other incidences where the SNP common allele provides one or two unique punitive TFBS while the minor alleles again provide several (*e.g.,* rs948988 in ADRBK1; rs4590656 in AKT3; rs11119982 in ATF3; rs8179673 in STAT4 and rs2238631 in TBXA2R). A near balance between SNP alleles in unique punitive TFBS can also be found in the table (*e.g.,* rs4370946 in ADRBK1; rs10157763 and rs2125230 in AKT3; rs3125289 in ATF3; rs6756667 in EPAS1; rs34357231 and rs3025039 in VEGFA). The minor allele of the SNP usually generates more punitive unique TFBS than the common allele (*e.g.,* rs948988 in ADRBK1; rs1412444 in LIPA and rs8179673 in STAT4). In fact, from this sampling of SNPs among the nine genes, there are 73 potential unique TFBS generated by the common alleles compared to 124 by the minor alleles (Table 2).

DISCUSSION

The possible relationship of these punitive unique TFBS to disease and sickness has previously been discussed for each gene in the accompanying references (Table 1). The use of rSNPs that are in LD within a gene to identify punitive TFBS can be illustrated with a few SNPs from these nine genes. The *ADRBK1* gene, which transcribes the GRK2 kinase, is an important regulator of beta-adrenergic signaling and plays a central role in heart failure (HF) pathology[32-34]. Two rSNPs in LD within the *ADRBK1* gene are rs948988 and rs4370946 whose minor alleles create punitive unique TFBS for ESR2 that is a binding site for the beta estrogen receptor which is expressed in blood monocytes and pulmonary epithelial cells (Tables 1-3). The ESR2 TFBS is not found with the common (rs948988 and rs4370946) alleles of the gene and may be related to HF. The NR3C1 TFBS for the glucocorticoid receptor which regulates carbohydrate, protein and fat metabolism is also only found with the minor alleles of these rSNPs (Tables 2 and 3) and should have an impact on HF. Other TFBS generated by the rs948988 minor allele of interest in HF might be the MYB and NFE2L.1:MAF TFs which are involved with hematopoietic progenitor cells and cell differentiation of erythrocytes as well as the rs4370946 common allele for the NRF1 TF which is involved with heme biosynthesis and mitochondrial DNA transcription and replication (Tables 2 and 3).

The type 2 demodkinase gene (*DIO2*) encodes a deiodinase that coverts the thyroid prohormone, thyroxine (T4), to the biologically active triiodothyronine (T3) where T3 plays an important role in the regulation of energy balance and glucose metabolism[35-38]. Two rSNPs in LD within the *DIO2* gene are rs225015 and rs225011 whose major alleles create unique punitive TFBS for TFs that are involved with energy balance and glucose metabolism (Tables 1-3). The ESRRα an alpha estrogen-related receptor that is involved with regulating thyroid hormone receptor genes while PPARα:RXRα and RXRα are involved with the regulation of adipocyte differentiation and glucose homeostasis (Tables 2 and 3). The minor allele of the rs225015 rSNP creates a unique punitive TFBS for the TCF7L2 TF whose protein is implicated in blood glucose homeostasis (Tables 2 and 3). The minor allele of the rs225011 rSNP creates a unique punitive TFBS for the PDX1 TF whose protein activates insulin, somatostatin, glucokinase, islet amyloid

Table 3 Transcriptional factors, protein name and their description or function

TF	Protein name	Transcriptional factor description/function
AR	Androgen receptor	The protein functions as a steroid-hormone activated transcription factor Upon binding the hormone ligand, the receptor dissociates from accessory proteins, translocates into the nucleus, dimerizes, and then stimulates transcription of androgen responsive genes. They are expressed in bone marrow, mammary gland, prostate, testicular and muscle tissues where they exist as dimers coupled to *Hsp90* and *HMGB* proteins
ARID3A	AT rich interactive domain 3A (BRIGHT-like)	This gene encodes a member of the AT-rich interaction domain family of DNA binding proteins
ARNT	Aryl hydrocarbon receptor nuclear translocator	Involved in the induction of several enzymes that participate in xenobiotic metabolism
ARNT:AHR	Hypoxia-inducible factor 1:Aryl hydrocarbon receptor nuclear translocator	The dimer alters transcription of target genes. Involved in the induction of several enzymes that participate in xenobiotic metabolism
ATF7	Activating Transcription Factor 7	Plays important functions in early cell signaling Has no intrinsic transcriptional activity, but activates transcription on formation of JUN or FOS heterodimers
ATOH1	Atonal homolog 1	Transcriptional regulator. Activates E box-dependent transcription in collaboration with TCF3/E47
BATF::JUN	Basic leucine zipper transcription factor, ATF-like Jun proto-oncogene	The protein encoded by this gene is a nuclear basic leucine zipper protein that belongs to the AP-1/ATF superfamily of transcription factors. The leucine zipper of this protein mediates dimerization with members of the Jun family of proteins. This protein is thought to be a negative regulator of AP-1/ATF transcriptional events
BRCA1	Breast cancer 1, early onset	This gene encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability, and it also acts as a tumor suppressor
CEBPA	CCAAT/enhancer binding protein, alpha	CCAAT/enhancer binding protein is a DNA-binding protein that recognizes two different motifs: the CCAAT homology common to many promoters and the enhanced core homology common to many enhancers
CREB1	cAMP responsive element binding protein 1	Phosphorylation-dependent transcription factor that stimulates transcription upon binding to the DNA cAMP response element, a sequence present in many viral and cellular promoters
CRX	Cone-rod homeobox	The protein encoded by this gene is a photoreceptor-specific transcription factor which plays a role in the differentiation of photoreceptor cells. This homeodomain protein is necessary for the maintenance of normal cone and rod function
CTCF	CCCTC-binding factor (zinc finger protein)	This gene is a member of the BORIS + CTCF gene family and encodes a transcriptional regulator protein with 11 highly conserved zinc finger domains. This nuclear protein is able to use different combinations of the zinc finger domains to bind different DNA target sequences and proteins
E2F1-6	E2F transcription factors 1-6	The protein encoded by this gene is a member of the E2F family of transcription factors. The E2F family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses. The E2F proteins contain several evolutionally conserved domains found in most members of the family. These domains include a DNA binding domain, a dimerization domain which determines interaction with the differentiation regulated transcription factor proteins, a transactivation domain enriched in acidic amino acids, and a tumor suppressor protein association domain which is embedded within the transactivation domain
EBF1	Transcription factor COE1	EBF1 has been shown to interact with ZNF423 and CREB binding proteins
EGR1	Early growth response 1	The protein encoded by this gene belongs to the EGR family of C2H2-type zinc-finger proteins. It is a nuclear protein and functions as a transcriptional regulator. The products of target genes it activates are required for differentiation and mitogenesis
EGR2	Early growth response 2	The protein encoded by this gene is a transcription factor with three tandem C2H2-type zinc fingers Sequence-specific DNA-binding transcription factor
EHF	Ets homologous factor	This gene encodes a protein that belongs to an erythroblast transformation-specific transcription factor subfamily characterized by epithelial-specific expression. The encoded protein acts as a transcriptional repressor and may be involved in epithelial differentiation and carcinogenesis
ELF1	E74-like factor 1 (ets domain transcription factor)	The encoded protein is primarily expressed in lymphoid cells and acts as both an enhancer and a repressor to regulate transcription of various genes
ELF5	E74-like factor 5	A member of an epithelium-specific subclass of the Ets Transcription factor family
ELK1	ELK1, member of ETS oncogene family	This gene is a member of the Ets family of transcription factors and of the ternary complex factor subfamily. The protein encoded by this gene is a nuclear target for the ras-raf-MAPK signaling cascade
ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)	This gene is a member of the Ets family of transcription factors and of the ternary complex factor subfamily. Proteins of the ternary complex factor subfamily form a ternary complex by binding to the serum response factor and the serum response element in the promoter of the c-fos proto-oncogene
EN1	Engrailed homeobox 1	Homeobox-containing genes are thought to have a role in controlling development

ERG	v-ets avian erythroblastosis virus E26 oncogene homolog	This gene encodes a member of the erythroblast transformation-specific family of transcriptions factors. All members of this family are key regulators of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis
ESR2	Estrogen receptor beta	Estrogen receptor β is a member of the family of estrogen receptors and the superfamily of nuclear receptor transcription factors and is expressed by many tissues including blood monocytes and tissue macrophages, colonic and pulmonary epithelial cells
ESRRA	Estrogen-related receptor alpha	This nuclear receptor acts as a site-specific transcription regulator and has been also shown to interact with estrogen and the transcripton factor TFIIB by direct protein-protein contact. The binding and regulatory activities of this protein have been demonstrated in the regulation of a variety of genes including lactoferrin, osteopontin, medium-chain acyl coenzyme A dehydrogenase and thyroid hormone receptor genes
ETS1	Protein C-ets-1	The protein encoded by this gene belongs to the erythroblast transformation-specific family of transcription factors and has been shown to interact with TTRAP, UBE2I and Death associated protein
FEV	ETS oncogene family	It functions as a transcriptional repressor
FLI1	Fli-1 proto-oncogene, ETS transcription factor	Sequence-specific transcriptional activator
FOS	FBJ murine osteosarcoma viral oncogene homolog	The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic cell death
FOSL1 and 2	FOS-like antigen 1 and 2	GO annotations related to this gene include RNA polymerase II regulatory region sequence-specific DNA binding and sequence-specific DNA binding transcription factor activity
FOXA1	Forkhead box A1	Transcription factor that is involved in embryonic development, establishment of tissue-specific gene expression and regulation of gene expression in differentiated tissues. Is thought to act as a "pioneer" factor opening the compacted chromatin for other proteins through interactions with nucleosomal core histones and thereby replacing linker histones at target enhancer and/or promoter sites Involved in the development of multiple endoderm-derived organ systems such as liver, pancreas, lung and prostate. Modulates the transcriptional activity of nuclear hormone receptors
FOXA2	Forkhead box A2	Involved in embryonic development, establishment of tissue-specific gene expression and regulation of gene expression in differentiated tissues
FOXC1	Forkhead box C1	An important regulator of cell viability and resistance to oxidative stress in the eye
FOXL1	Forkhead box L1	Transcription factor required for proper proliferation and differentiation in the gastrointestinal epithelium. Target gene of the hedgehog signaling pathway
FOXO1	Forkhead Box O1	Transcription factor that is the main target of insulin signaling and regulates metabolic homeostasis in response to oxidative stress
FOXO3	Forkhead Box O3	This gene belongs to the forkhead family of transcription factors which are characterized by a distinct forkhead domain. This gene likely functions as a trigger for apoptosis through expression of genes necessary for cell death
FOXP1	Forkhead box P1	This gene belongs to subfamily P of the forkhead box transcription factor family. Forkhead box transcription factors play important roles in the regulation of tissue- and cell type-specific gene transcription during both development and adulthood. Transcriptional repressor. It plays an important role in the specification and differentiation of lung epithelium
FOXQ1	Forkhead box Q1	This gene belongs to the forkhead family of transcription factors which is characterized by a distinct DNA-binding forkhead domain. Plays a role in hair follicle differentiation
GABPA	GA-binding protein alpha chain	One of three GA-binding protein transcription factor subunits which functions as a DNA-binding subunit which shares identity with a subunit encoding the nuclear respiratory factor 2 gene and is likely involved in activation of cytochrome oxidase expression and nuclear control of mitochondrial function
GATA1	GATA binding protein 1	The protein plays an important role in erythroid development by regulating the switch of fetal hemoglobin to adult hemoglobin
GATA2	GATA binding protein 2	A member of the GATA family of zinc-finger transcription factors that are named for the consensus nucleotide sequence they bind in the promoter regions of target genes and play an essential role in regulating transcription of genes involved in the development and proliferation of hematopoietic and endocrine cell lineages
GATA3	GATA binding protein 3	Plays an important role in endothelial cell biology
GFI	Growth factor independent 1 transcription repressor	This gene encodes a nuclear zinc finger protein that functions as a transcriptional repressor. This protein plays a role in diverse developmental contexts, including hematopoiesis and oncogenesis. It functions as part of a complex along with other cofactors to control histone modifications that lead to silencing of the target gene promoters
GMEB2	Glucocorticoid modulatory element binding protein 1	This gene is a member of KDWK gene family. The product of this gene associates with GMEB1 protein, and the complex is essential for parvovirus DNA replication

HAND1: TCFE2α	Heart- and neural-crest derivatives-expressed protein 1: Transcription factor E2A	Hand1 belongs to the basic helix-loop-helix family of transcription factors The *Tcfe2a* gene encodes the transcription factor E2A, a member of the "class I" a family of basic helix-loop-helix transcription factors (also known simply as "E-proteins"). The transcription factor E2A controls the initiation of B lymphopoiesis
HIC1	Hypermethylated in cancer 1	This gene functions as a growth regulatory and tumor repressor gene
HIF1A:ARNT	Hypoxia-inducible factor 1: Aryl hydrocarbon receptor nuclear translocator	HIF1 is a homodimeric basic helix-loop-helix structure composed of HIF1a, the alpha subunit, and the aryl hydrocarbon receptor nuclear translocator (Arnt), the beta subunit. The protein encoded by HIF1 is a Per-Arnt-Sim transcription factor found in mammalian cells growing at low oxygen concentrations. It plays an essential role in cellular and systemic responses to hypoxia
HLTF	Helicase-like transcription factor	Member of the SWItch/Sucrose Non Fermentable family which have helicase and ATPase activities and are thought to regulate transcription of certain genes by altering the chromatin
HNF1A	Hepatocyte nuclear factor 1 homeobox A	Transcriptional activator that regulates the tissue specific expression of multiple genes, especially in pancreatic islet cells and in liver
HNF1B	HNF1 homeobox B	This gene encodes a member of the homeodomain-containing superfamily of transcription factors. The protein binds to DNA as either a homodimer, or a heterodimer with the related protein hepatocyte nuclear factor 1-alpha. The gene has been shown to function in nephron development, and regulates development of the embryonic pancreas
HNF4α	Hepatocyte nuclear factor 4, alpha	The protein encoded by this gene is a nuclear transcription factor which binds DNA as a homodimer. The encoded protein controls the expression of several genes, including hepatocyte nuclear factor 1 alpha, a transcription factor which regulates the expression of several hepatic genes. This gene may play a role in development of the liver, kidney, and intestines
HNF4γ	Hepatocyte nuclear factor 4, gamma	Steroid hormone receptor activity and sequence-specific DNA binding transcription factor activity. An important paralog of this gene is RXRA
HOXA5	Homeobox protein Hox-A5	DNA-binding transcription factor which may regulate gene expression, morphogenesis, and differentiation
ISNM1	Insulinoma-associated 1	Insulinoma-associated 1 gene is intronless and encodes a protein containing both a zinc finger DNA-binding domain and a putative prohormone domain. This gene is a sensitive marker for neuroendocrine differentiation of human lung tumors
IRF1,2	Interferon regulatory factor	Members of the interferon regulatory transcription factor family that contain a conserved N-terminal region of about 120 amino acids, which folds into a structure that binds specifically to the interferon consensus sequence
JDP2	Jun dimerization protein 2	Component of the AP-1 transcription factor that represses transactivation mediated by the Jun family of proteins. Involved in a variety of transcriptional responses associated with AP-1 such as UV-induced apoptosis, cell differentiation, tumorigenesis and antitumogeneris
JUN	Jun Proto-Oncogene	This gene is the putative transforming gene of avian sarcoma virus 17. It encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression
JUND	Jun D proto-oncogene	The protein encoded by this intronless gene is a member of the JUN family, and a functional component of the AP1 transcription factor complex. This protein has been proposed to protect cells from p53-dependent senescence and apoptosis
JUN::FOS	Jun proto-oncogene FBJ murine osteosarcoma viral oncogene homolog	Promotes activity of NR5A1 when phosphorylated by HIPK3 leading to increased steroidogenic gene expression upon cAMP signaling pathway stimulation Has a critical function in regulating the development of cells destined to form and maintain the skeleton. It is thought to have an important role in signal transduction, cell proliferation and differentiation
KLF1	Kruppel-like factor 1 (erythroid)	Transcription regulator of erythrocyte development that probably serves as a general switch factor during erythropoiesis. Is a dual regulator of fetal-to-adult globin switching
KLF4	Krueppel-like factor 4	Transcription factor that can act both as activator and as repressor. Regulates the expression of key transcription factors during embryonic development
KLF5	Krueppel-like factor 5	This gene encodes a member of the Kruppel-like factor subfamily of zinc finger proteins. The encoded protein is a transcriptional activator that binds directly to a specific recognition motif in the promoters of target genes. This protein acts downstream of multiple different signaling pathways and is regulated by post-translational modification. It may participate in both promoting and suppressing cell proliferation. Expression of this gene may be changed in a variety of different cancers and in cardiovascular disease. Alternative splicing results in multiple transcript variants
MAX	MYC associated factor X	The protein encoded by this gene is a member of the basic helix-loop-helix leucine zipper family of transcription factors

MAFK	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K	Since they lack a putative transactivation domain, the small Mafs behave as transcriptional repressors when they dimerize among themselves. However, they seem to serve as transcriptional activators by dimerizing with other (usually larger) basic-zipper proteins and recruiting them to specific DNA-binding sites
MEF2A	Myocyte enhancer factor 2A	The protein encoded by this gene is a DNA-binding transcription factor that activates many muscle-specific, growth factor-induced, and stress-induced genes. Mediates cellular functions not only in skeletal and cardiac muscle development, but also in neuronal differentiation and survival
MGA	MGA, MAX Dimerization Protein	Functions as a dual-specificity transcription factor, regulating the expression of both MAX-network and T-box family target genes. Functions as a repressor or an activator
MYB	Myb proto-oncogene protein	This gene encodes a transcription factor that is a member of the MYB family of transcription factor genes. Transcriptional activator and plays an important role in the control of proliferation and differentiation of hematopoietic progenitor cells
MYC	v-myc myelocytomatosis viral oncogene homolog	The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	This gene is a member of the MYC family and encodes a protein with a basic helix-loop-helix domain. Amplification of this gene is associated with a variety of tumors, most notably neuroblastomas
MZF1_1-4	Myeloid zinc finger 1	Binds to target promoter DNA and functions as transcription regulator. May be one regulator of transcriptional events during hemopoietic development. Isoforms of this protein have been shown to exist at protein level
NFATC2	Nuclear factor of activated T-cells, cytoplasmic 2	This protein is present in the cytosol and only translocates to the nucleus upon T cell receptor stimulation, where it becomes a member of the nuclear factors of activated T cells transcription complex
NFIA	Nuclear Factor I/A	Recognizes and binds the palindromic sequence 5-TTGGCNNNNNGCCAA-3 present in viral and cellular promoters transcription and replication and in the origin of replication of adenovirus type 2. These proteins are individually capable of activating transcription and replication
NFIC	Nuclear factor 1 C-type	Recognizes and binds the palindromic sequence 5'-TTGGCNNNNNGCCAA-3' present in viral and cellular promoters and in the origin of replication of adenovirus type 2. These proteins are individually capable of activating transcription and replication
NFE2::MAF	Nuclear factor, erythroid 2 V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog	Regulates erythroid and megakaryocytic maturation and differentiation. Plays a role in all aspects of hemoglobin production from globin and heme synthesis to procurement of iron. When overexpressed, represses anti-oxidant response element-mediated transcription
NFE2L1: MAFG	Nuclear factor erythroid 2-related factor 1 Transcription factor MafG	Nuclear factor erythroid 2-related factor coordinates the up-regulation of cytoprotective genes via the antioxidant response element. MafG is a ubiquitously expressed small maf protein that is involved in cell differentiation of erythrocytes. It dimerizes with P45 NF-E2 protein and activates expression of a and b-globin
NFIL3	Nuclear factor, interleukin 3 regulated	Expression of interleukin-3 (MIM 147740) is restricted to activated T cells, natural killer cells, and mast cell lines
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	natural killer-kappa-B is a pleiotropic transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis
NFYB	Nuclear transcription factor Y, beta	The protein encoded by this gene is one subunit of a trimeric complex, forming a highly conserved transcription factor that binds with high specificity to CCAAT motifs in the promoter regions in a variety of genes. This gene product, subunit B, forms a tight dimer with the C subunit, a prerequisite for subunit A association. The resulting trimer binds to DNA with high specificity and affinity. Subunits B and C each contain a histone-like motif
NHLH1	Nescient helix loop helix 1	The helix-loop-helix proteins are a family of putative transcription factors, some of which have been shown to play an important role in growth and development of a wide variety of tissues and species
NKX2-5	Natural killer 3 homeobox 2	This gene encodes a member of the natural killer family of homeobox-containing proteins Transcriptional repressor that acts as a negative regulator of chondrocyte maturation
NKX3-2	Natural killer 3 homeobox 2	This gene encodes a member of the natural killer family of homeobox-containing proteins Transcriptional repressor that acts as a negative regulator of chondrocyte maturation
NR1H3:RXRa	Nuclear Receptor Subfamily 1, Group H, Member 3 Retinoid X receptor, alpha	The protein encoded by this gene belongs to the NR1 subfamily of the nuclear receptor superfamily The NR1 family members are key regulators of macrophage function, controlling transcriptional programs involved in lipid homeostasis and inflammation. This protein is highly expressed in visceral organs, including liver, kidney and intestine. It forms a heterodimer with retinoid X receptor, and regulates expression of target genes containing retinoid response elements Studies in mice lacking this gene suggest that it may play an important role in the regulation of cholesterol homeostasis

NR2C2	Nuclear receptor subfamily 2, group C, member 2	Orphan nuclear receptor that can act as a repressor or activator of transcription. An important repressor of nuclear receptor signaling pathways such as retinoic acid receptor, retinoid X, vitamin D3 receptor, thyroid hormone receptor and estrogen receptor pathways
NR2E3	Nuclear receptor subfamily 2, group E, member 3	This protein is part of a large family of nuclear receptor transcription factors involved in signaling pathways
NR2F1 (COUP)	Nuclear receptor subfamily 2, group F, member 1	Binds to the ovalbumin promoter and, in conjunction with another protein (S300-II) stimulates initiation of transcription. Binds to both direct repeats and palindromes of the 5'-AGGTCA-3' motif. An important paralog of this gene is RXRA
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	Glucocorticoids regulate carbohydrate, protein and fat metabolism, modulate immune responses through suppression of chemokine and cytokine production and have critical roles in constitutive activity of the CNS, digestive, hematopoietic, renal and reproductive systems
NR4A2	Nuclear receptor subfamily 4, group A, member 2	Transcriptional regulator which is important for the differentiation and maintenance of meso-diencephalic dopaminergic neurons during development
NRF1	Nuclear respiratory factor 1	This gene encodes a protein that homodimerizes and functions as a transcription factor which activates the expression of some key metabolic genes regulating cellular growth and nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication
NRL	Neural Retina Leucine Zipper	This gene encodes a basic motif-leucine zipper transcription factor of the Maf subfamily. The encoded protein is conserved among vertebrates and is a critical intrinsic regulator of photoceptor development and function
PAX2	Paired box gene 2	Probable transcription factor that may have a role in kidney cell differentiation
PDX1	Pancreatic and duodenal homeobox 1	Activates insulin, somatostatin, glucokinase, islet amyloid polypeptide and glucose transporter type 2 gene transcription. Particularly involved in glucose-dependent regulation of insulin gene transcription
PPARγ:RXRα	Peroxisome proliferator-activated receptor gamma Retinoid X receptor, alpha	Peroxisome proliferator-activated receptor gamma is a member of the nuclear receptor family of ligand-activated transcription factors that heterodimerize with the retinoic X receptor to regulate gene expression. Peroxisome proliferator-activated receptor gamma is located primarily in the adipose tissue, lymphoid tissue, colon, liver and heart and is thought to regulate adipocyte differentiation and glucose homeostasis
RXRa	Retinoid X receptor, alpha	Retinoid X receptors and retinoic acid receptors, are nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid-mediated gene activation
RFX5	Regulatory factor X, 5	Activates transcription from class II MHC promoters. Recognizes X-boxes. Mediates cooperative binding between RFX and natural killer-Y. RFX binds the X1 box of MHC-II promoters
RUNX1	Runt-related transcription factor 1	Heterodimeric transcription factor that binds to the core element of many enhancers and promoters. The protein encoded by this gene represents the alpha subunit of core binding factor and is thought to be involved in the development of normal hematopoiesis
SOX6	SRY (sex determining region Y)-box 6	The encoded protein is a transcriptional activator that is required for normal development of the central nervous system, chondrogenesis and maintenance of cardiac and skeletal muscle cells
SOX9	SRY (sex determining region Y)-box 9	The protein encoded by this gene recognizes the sequence CCTTGAG along with other members of the involved in chondrogenesis by acting as a transcription factor for these genes
SOX10	SRY (sex determining region Y)-box 10	This gene encodes a member of the SRY-related HMG-box family of transcription factors involved in the regulation of embryonic development and in the determination of the cell fate
SOX17	SRY (sex determining region Y)-box 17	Acts as transcription regulator that binds target promoter DNA and bends the DNA
SP1	Specificity Protein 1	Can activate or repress transcription in response to physiological and pathological stimuli. Regulates the expression of a large number of genes involved in a variety of processes such as cell growth, apoptosis, differentiation and immune responses
SP2	Specificity Protein 2	This gene encodes a member of the Sp subfamily of Sp/XKLF transcription factors. Sp family proteins are sequence-specific DNA-binding proteins characterized by an amino-terminal trans-activation domain and three carboxy-terminal zinc finger motifs. This protein contains the least conserved DNA-binding domain within the Sp subfamily of proteins, and its DNA sequence specificity differs from the other Sp proteins. It localizes primarily within subnuclear foci associated with the nuclear matrix, and can activate or in some cases repress expression from different promoters
SPIB	Transcription factor Spi-B	SPI1 and SPIB are members of a subfamily of erythroblast transformation-specific transcription factors; erythroblast transformation-specific proteins share a conserved erythroblast transformation-specific domain that mediates specific DNA binding SPIB and SPI1 bind to a purine-rich sequence, the PU box (5-prime-GAGGAA-3-)
SPI1	Spleen focus forming virus proviral integration oncogene spi1	This gene encodes an erythroblast transformation-specific-domain transcription factor that activates gene expression during myeloid and B-lymphoid cell development
SPZ1		This gene encodes a basic helix-loop-helix-zip transcription factor which functions in the mitogen-activate protein kinase signaling pathway

SRY	Sex determining region Y	Transcriptional regulator that controls a genetic switch in male development
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	Signal transducer and transcription activator that mediates cellular responses to interleukins, KITLG/SCF and other growth factors
STAT5A: STAT5B	Signal transducer and activator of transcription 5A and transcription 5B	Carries out a dual function: signal transduction and activation of transcription Regulates the expression of milk proteins during lactation
TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	This gene encodes a high mobility group box-containing transcription factor that plays a key role in the Wnt signaling pathway. The protein has been implicated in blood glucose homeostasis
TEAD1	TEA Domain Family Member 1	This gene encodes a ubiquitous transcriptional enhancer factor that is a member of the TEA/ATTS domain family. This protein directs the transactivation of a wide variety of genes and, in placental cells, also acts as a transcriptional repressor
TFAP2a	Activator protein 2	The AP2a protein acts as a sequence specific DNA-binding transcription factor recognizing and binding to the specific DNA sequence and recruiting transcription machinery
THAP1	THAP domain containing, apoptosis associated protein 1	DNA-binding transcription regulator that regulates endothelial cell proliferation and G1/S cell-cycle progression
USF1	Upstream transcription factor 1	This gene encodes a member of the basic helix-loop-helix leucine zipper family, and can function as a cellular transcription factor. The encoded protein can activate transcription through pyrimidine-rich initiator (Inr) elements and E-box motifs
YY1	YY1 transcription factor	YY1 is a ubiquitously distributed transcription factor belonging to the GLI-Kruppel class of zinc finger proteins. The protein is involved in repressing and activating a diverse number of promoters. YY1 may direct histone deacetylases and histone acetyltransferases to a promoter in order to activate or repress the promoter, thus implicating histone modification in the function of YY1
ZEB1	Zinc finger E-box-binding homeobox 1	A member of the delta-EF1 (TCF8)/Zfh1 family of 2-handed zinc finger/homeodomain proteins and interacts drosophila mothers against decapentaplegic proteins with receptor-mediated, activated full-length activated full-length drosophila mothers against decapentaplegic protein
ZNF263	Zinc finger protein 263	Might play an important role in basic cellular processes as a transcriptional repressor. An important paralog to ZNF496
ZNF354C	Zinc finger protein 354C	May function as a transcription repressor

polypeptide and glucose transporter type 2 gene transcription (Tables 2 and 3).

The thromboxane A2 receptor (*TBXA2R*) gene is a member of the seven-transmembrane G-protein-coupled receptor super family, which interacts with intracellular G proteins, regulates different downstream signaling cascades, and induces many cellular responses including the intracellular calcium influx, cell migration and proliferation as well as apoptosis[39]. Two rSNPs in LD within the *TBXA2R* gene are rs2238631 and rs2238634 whose minor alleles create unique punitive TFBS for TFs that are involved in signaling cascades and apoptosis (Tables 1-3). The ELK1 and SPZ1 TFs are involved with the ras-raf-MAPK signaling cascade while the ETS1 TF is involved with cell death (Tables 2 and 3). NR2E3 is part of a large family of nuclear receptor TFs involved in signaling pathways (Tables 2 and 3).

The other six genes can be analyzed in the same manner to identify punitive TFBS created by the rSNP alleles of these genes (Tables 2 and 3). What a change in the rSNP alleles can do, is to alter the DNA landscape around the SNP for potential TFs to attach and regulate a gene. This change in the DNA landscape can alter gene regulation which in turn can result in a change of a biological process or signaling pathway resulting in disease or illness. The process laid out in this report is a convenient way of identifying potential TFBS created by rSNP alleles that have been found to be significantly associated with disease or sickness. Any potential alterations in TFBS obtained by computational analyses need to be verified by protein/DNA electrophoretic mobility gel shift assays and gene expression studies[40]. CHIP-seq[41] experiments have become the standard method of validating TFBS and studying gene regulation[42-44].

In conclusion, SNPs in the regulatory region of a gene can alter the DNA landscape for TFs resulting in TFBS changes. Consequently, alterations in TF binding can affect gene regulation. Examples of this for nine genes are presented in this report where SNP alleles will either have no effect on TF binding or each allele will create unique punitive TFBS and alter a TFs ability to bind the DNA and regulate the gene.

COMMENTS

Background

Transcriptional factors (TFs) bind the DNA near a gene at transcriptional factor binding sites (TFBS) in order to regulate the gene. Single nucleotide polymorphisms (SNPs) that occur in the TFBS can alter the TFs ability to bind the DNA and thereby affect gene regulation. Such regulatory (r)SNPs have been associated with human disease and sickness. In this report, the alteration of TFBS created by rSNP alleles associated with disease has been documented for nine human genes. Sometimes the rSNP alleles will have no effect on the TFBS and not change the TF ability to bind the DNA. Other times each allele will create unique punitive TFBS that alter the TFs ability to regulate the gene.

Research frontiers

This article addresses an emerging concept in understanding how rSNPs which are significantly associated with disease can alter the TFBS for TFs that regulate a gene.

Innovations and breakthroughs

TFBS alteration by rSNPs is a newly emerging field of research and provides a different direction in examining changes in gene regulation resulting in human

disease and sickness.

Applications

Given the great diversity of punitive unique TFBS generated by each allele of a rSNP, the author suspects that alterations in TFBS affect how well a gene is expressed. The outcome may result in disease or sickness. The methods outlined in the article should be applied to all rSNPs that are associated with disease or sickness of a regulatory nature.

Terminology

rSNP: A regulatory single nucleotide polymorphism that affects gene expression; TF: Transcriptional factor that is involved with regulating a gene; TFBS: Transcription factor DNA binding site in the regulatory region of a gene; Unique TFBS: A TFBS created by one rSNP allele and not the alternate allele.

Peer-review

This study is technically well performed and a very interesting result. The interpretation was also sound. The report applied a computational approach to predict functional rSNPs in TFBS, focusing on several genes published earlier. Computational modeling and analysis for functional prediction is one of the approaches recently developed, particularly to address GWAS findings.

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ORIGINAL ARTICLE

Basic Study

P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes

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Abstract

AIM

To determine if activation of the ATP-gated P2X7 receptor channel induces phosphatidylserine (PS) exposure in erythrocytes from multiple dog breeds.

METHODS

Peripheral blood was collected from 25 dogs representing 13 pedigrees and seven crossbreeds. ATP-induced PS exposure on canine erythrocytes *in vitro* was assessed using a flow cytometric Annexin V binding assay.

RESULTS

ATP induced PS exposure in erythrocytes from all dogs

studied. ATP caused PS exposure in a concentration-dependent manner with an EC_{50} value of 395 μmol/L. The non-P2X7 agonists, ADP or AMP, did not cause PS exposure. The P2X7 antagonist, AZ10606120, but not the P2X1 antagonist, NF449, blocked ATP-induced PS exposure.

CONCLUSION
The results indicate that ATP induces PS exposure in erythrocytes from various dog breeds and that this process is mediated by P2X7 activation.

Key words: Adenosine triphosphate; Dog; P2X1 receptor; P2X7 receptor; Phospholipid; Purinergic receptor; Red blood cells

Core tip: Phosphatidylserine (PS) exposure in erythrocytes has potential roles in erythrocyte clearance and thrombus formation. Activation of the ATP-gated P2X7 receptor channel induces PS exposure in human erythrocytes, but whether this process occurs in erythrocytes from other mammals remained hitherto unknown. The current study shows that extracellular ATP causes PS exposure in dog erythrocytes from 13 pedigrees and seven crossbreeds. Notably, the current study shows that this process is mediated by P2X7 activation. These results suggest that P2X7-mediated PS exposure on erythrocytes may have important roles in red blood cell biology in dogs.

Faulks M, Kuit TA, Sophocleous RA, Curtis BL, Curtis SJ, Jurak LM, Sluyter R. P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes. *World J Hematol* 2016; 5(4): 88-93 Available from: URL: http://www.wjgnet.com/2218-6204/full/v5/i4/88.htm DOI: http://dx.doi.org/10.5315/wjh.v5.i4.88

INTRODUCTION

Exposure of the plasma membrane lipid, phosphatidylserine (PS), to the outer leaflet is an important physiological and pathophysiological signal[1]. In erythrocytes, PS exposure serves emerging roles in the clearance of senescent, damaged and diseased erythrocytes from the circulation[2]. Moreover, PS exposure can serve as a substrate for thrombin formation and incorporation of erythrocytes into thrombi[3]. PS exposure also serves as a parameter for stored erythrocyte integrity[4] and may be important in the removal of such cells following transfusion[5]. Thus, it remains important to understand the mechanisms by which PS becomes exposed on the surface of erythrocytes.

The P2X7 receptor is a trimeric ligand-gated channel activated by extracellular ATP[6] at concentrations at least 10-fold greater than that required for other purinergic receptors[7]. Functional P2X7 has been reported in humans, dogs, rodents and other species[8]. P2X7 and other purinergic receptors, namely P2X1, P2Y1 and P2Y13, are present on the plasma membrane of erythrocytes[9]. P2X7 activation induces PS exposure in human erythrocytes[10,11], but it remains unknown if P2X7 activation mediates PS exposure in erythrocytes from other species. ATP can induce PS exposure in erythrocytes obtained from English springer spaniels[12], but whether this process occurs in other dog breeds and whether it is mediated by P2X7 activation remains to be determined. P2X7, however, is present in leukocytes from various dog breeds[13,14] suggesting that P2X7 activation may mediate PS exposure in canine erythrocytes.

Using a flow cytometric Annexin V binding assay, the current study aimed to determine if ATP induces PS exposure in erythrocytes from multiple dog breeds and whether this process is mediated by P2X7 activation.

MATERIALS AND METHODS

Materials

Nucleotides were from Sigma Chemical Co. (St. Louis, MO). AZ10606120 was from Tocris Bioscience (Ellisville, MO). NF499 was from Cayman Chemical (Ann Arbor, MI).

Blood samples

Peripheral blood was collected from either pedigree or crossbreed dogs into VACUETTE lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany). All samples were collected from privately owned dogs presenting at the Albion Park Veterinary Hospital (Albion Park, Australia), with informed consent of owners, and in accordance with and approval from the Animal and Human Ethics Committees of the University of Wollongong (Wollongong, Australia). The animal protocol was designed to minimize pain or discomfort to the animals, and conducted according to standard veterinary practices.

PS exposure assays

Erythrocytes from peripheral blood were isolated and resuspended in NaCl medium (147.5 mmol/L NaCl, 2.5 mmol/L KCl, 5 mmol/L glucose, 20 mmol/L HEPES, pH 7.4) at a final haematocrit of 2% as described[12]. Erythrocytes were then incubated in 96-well U-bottom plates (Greiner Bio-One) in the absence or presence of nucleotide (as indicated) for 24 h at 37 ℃/5% CO_2. In some experiments, erythrocytes were pre-incubated in the absence or presence of AZ10606120 or NF449 for 15 min at 37 ℃ prior to ATP addition. Following nucleotide incubation, 20 μL of resuspended erythrocytes were washed once in 1 mL Annexin V Binding Buffer (BioLegend, San Jose, CA) (450 × g for 3 min) and labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BioLegend, San Diego, CA) according to the manufacturer's instructions. Data was collected using a BD (San Jose, CA) LSR II or LSRFortessa flow cytometer

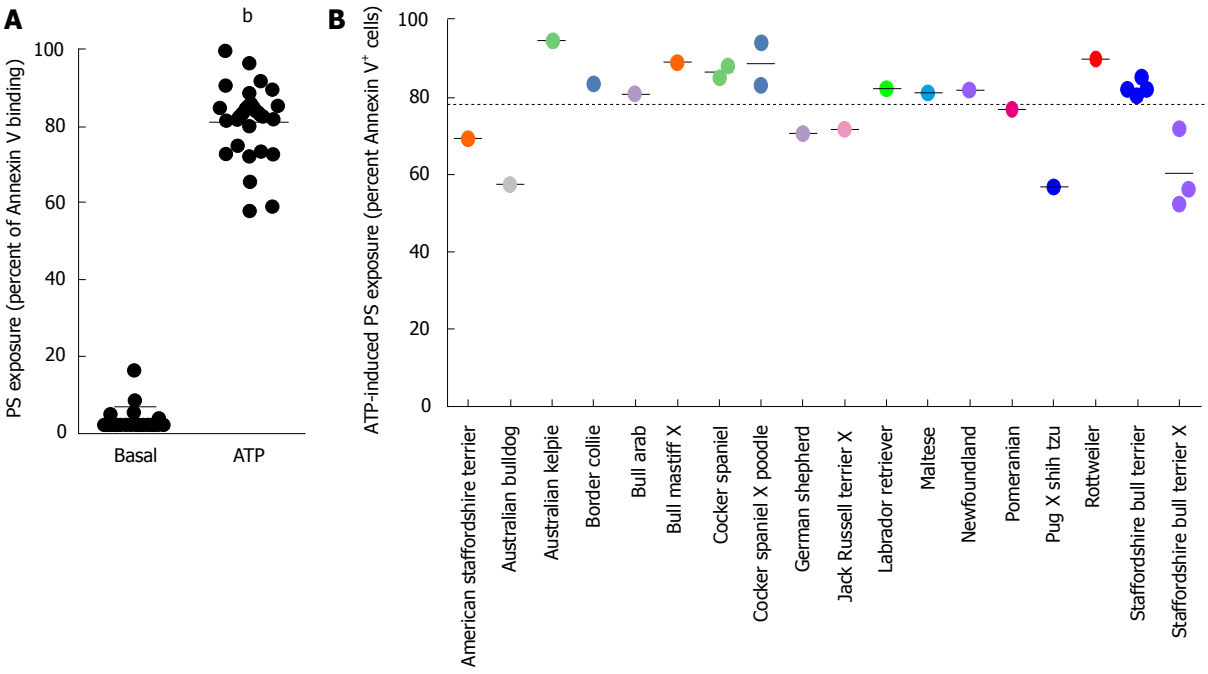

Figure 1 ATP induces phosphatidylserine exposure in erythrocytes from multiple dog breeds. Erythrocytes, from 17 pedigree and eight crossbreed (X) dogs, in NaCl medium were incubated for 24 h at 37 ℃ in the absence or presence of 1 mmol/L ATP. Cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. The bars represent group means (A and B). A: The symbols represent the percentage of Annexin V^+ erythrocytes, from each dog, following incubation in the absence (basal) or presence of ATP; $^bP < 0.0001$ ATP *vs* basal; B: The symbols represent the percentage of ATP-induced PS exposure in erythrocytes, from each dog, determined as the difference in the percentage of Annexin V^+ erythrocytes following incubation in the presence and absence of ATP. The broken line represents the mean ATP-induced PS exposure from all dogs. The symbols for Staffordshire bull terrier X represent a Staffordshire bull terrier and Australian kelpie cross, Staffordshire bull terrier and bull terrier cross, or a Staffordshire bull terrier cross. PS: Phosphatidylserine.

and FACSDiva software. The percentage of Annexin V^+ cells (PS exposure) was determined using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical analysis

Data is presented as mean ± SD. Statistical comparisons were performed using Prism 5 for Mac OS X (GraphPad Software, San Diego, CA). Differences between two or more groups were compared using a paired student's *t*-test or an ANOVA (using Tukey's multiple comparison test), respectively. Concentrations curves were fitted using the log(agonist) *vs* normalized response (variable slope) method.

RESULTS

To determine if ATP could induce PS exposure in erythrocytes in dog breeds other than English springer spaniels, erythrocytes, from 25 dogs representing 13 pedigrees and seven crossbreeds, were incubated in the absence or presence of ATP and the percent of Annexin V^+ cells (PS exposure) determined by flow cytometry. Incubation in the absence of ATP led to a mean PS exposure of 3.3% ± 3.2% (Figure 1A). In contrast, incubation with ATP caused a 25-fold increase in the mean PS exposure to 81.0% ± 10.4% (Figure 1A). Collectively, this resulted in an average ATP-induced PS exposure of 77.7% ± 11.8% (Figure 1B). Notably, ATP caused PS exposure in erythrocytes from all dogs studied (Figure 1B). ATP incubation also caused visible hemolysis compared to cells incubated in the absence of ATP (results not shown), but neither this nor other changes in erythrocyte morphology were investigated further.

To determine if P2X7 activation mediates exposure of PS in canine erythrocytes, erythrocytes were incubated with increasing concentrations of ATP and subsequent PS exposure assessed as described above. ATP induced PS exposure in a concentration-dependent manner with a maximum response at 2 mmol/L ATP and with an EC_{50} value of 395 ± 45 μmol/L (Figure 2A).

To further establish if P2X7 activation mediates PS exposure in canine erythrocytes, erythrocytes were incubated with ATP, as well as ADP and AMP, which do not activate canine P2X7[12,15]. Again ATP caused robust PS exposure in erythrocytes compared to erythrocytes incubated in the absence of nucleotide (Figure 2B). In contrast, ADP and AMP did not induce PS exposure in erythrocytes, with binding of Annexin V similar to that of erythrocytes incubated in the absence of nucleotide (Figure 2B).

Finally, canine erythrocytes were pre-incubated in the absence or presence of AZ10606120, which impairs canine P2X7[15], or NF449, which impairs human and rodent P2X1[16,17], prior to ATP incubation. Pre-incubation with AZ10606120 impaired ATP-induced PS exposure by 79%, while pre-incubation with NF449 had minimal effect on ATP-induced PS exposure (Figure 2C). Neither

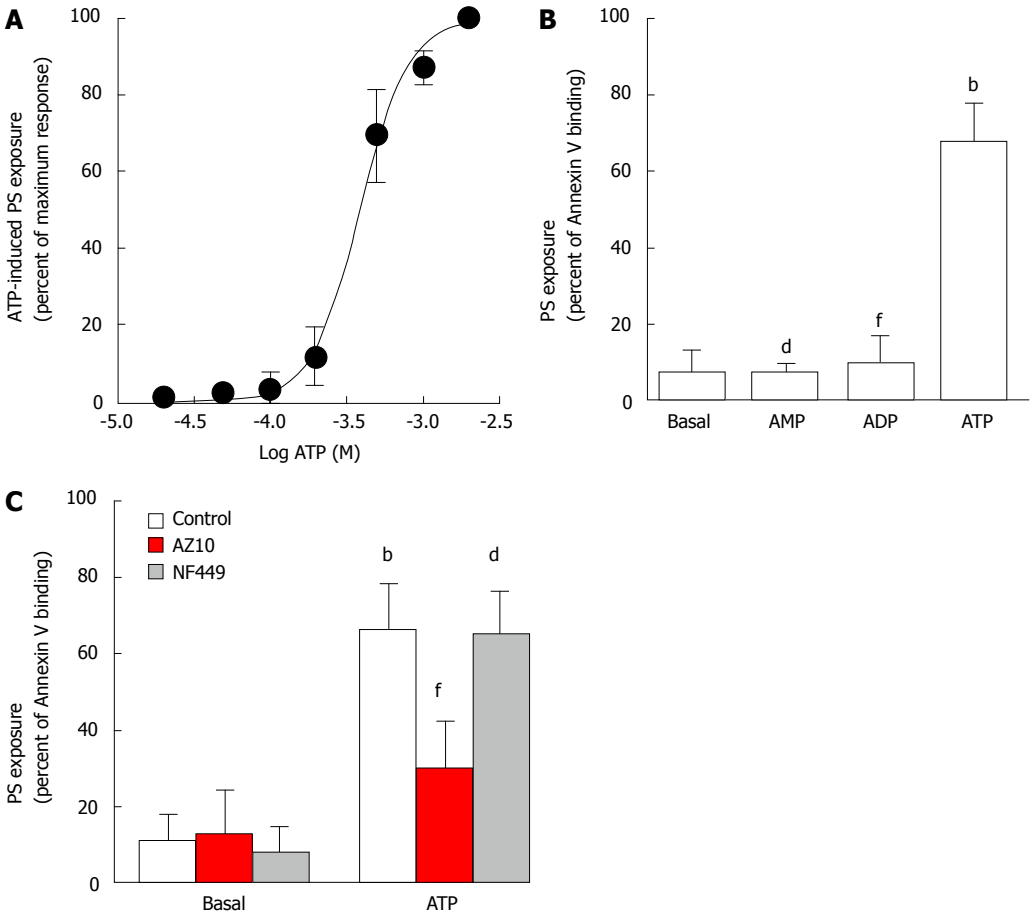

Figure 2 ATP induces phosphatidylserine exposure in canine erythrocytes in a concentration-dependent manner. Erythrocytes in NaCl medium were incubated for 24 h at 37 °C in the absence or presence of ATP (A) or 1 mmol/L nucleotide (B and C) (as indicated). Prior to ATP incubation, cells were pre-incubated for 15 min in the absence or presence of 10 μmol/L AZ10606120 (AZ10) or 10 μmol/L NF499 (C). Following nucleotide incubation, cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry (A-C). A: The data represent percent maximum response to 2 mmol/L ATP (mean ± SD, *n* = 4 dogs); B: The data represent mean ± SD (*n* = 5 dogs); $^{b}P < 0.001$ ATP *vs* basal; $^{d}P < 0.001$ AMP *vs* ATP; $^{f}P < 0.001$ ADP *vs* ATP; C: The data represent mean ± SD (*n* = 5 dogs); $^{b}P < 0.001$ ATP *vs* basal; $^{d}P < 0.001$ ATP with NF449 *vs* NF449; $^{f}P < 0.001$ ATP with AZ10606120 *vs* ATP.

AZ10606120 nor NF449 affected PS exposure in the absence of ATP (Figure 2C).

DISCUSSION

The current study demonstrated that ATP induces PS exposure in erythrocytes from 25 dogs representing 13 pedigrees and seven crossbreeds. On average, ATP caused PS exposure on 78% of erythrocytes from these dogs. This value is similar to that of ATP-induced PS exposure previously observed in erythrocytes from English springer spaniels (88%)[12]. Combined, these data indicate that ATP can induce PS exposure in erythrocytes from multiple dog breeds and suggests that this is likely to be a common phenomenon in all breeds of dogs. Moreover, these data confirm that ATP-induced PS exposure in canine erythrocytes is about six-fold greater than that observed for ATP-induced PS exposure in human erythrocytes[12], which corresponds to the increased expression and activity of P2X7 in canine erythrocytes compared to human erythrocytes[12,18].

Similar to human erythrocytes[10,11], the current study also demonstrates that ATP-induced PS exposure in canine erythrocytes is predominately mediated by P2X7 activation. First, the EC_{50} value for ATP-induced PS exposure (395 μmol/L) is similar to that observed for native and recombinant canine P2X7-mediated cation fluxes in English springer spaniel erythrocytes[12,18] and transfected HEK-293 cells[15,19], respectively; second, the non-P2X7 agonists, ADP and AMP, did not cause PS exposure; last, the P2X7 antagonist, AZ10606120, but not the P2X1 antagonist, NF449, impaired ATP-induced PS exposure. It should be noted that blockade with AZ10606120 was not complete indicating that either other purinergic receptors have an additional role in this process, or that AZ10606120 has limited efficacy in the conditions tested and that P2X7 remains solely responsible for ATP-induced PS exposure in canine erythrocytes. The latter is supported by at least three points. First, the concentration response curve for ATP-induced PS exposure revealed a simple, not biphasic, sigmoidal curve suggesting involvement of only one purinergic receptor subtype. Second, ATP concentrations below 100 μmol/L, which are sufficient to activate other ATP-responsive purinergic receptors[7], failed to cause PS exposure. Last, ADP, which can activate P2X1, P2Y1 and

P2Y13, but not P2X7[7], all of which are present in human or rodent erythrocytes[20-22], did not induce PS exposure.

It remains unknown why the relative amounts of P2X7 differ between canine and human erythrocytes, but we have previously speculated[12] that this difference may be due to alterations in the proteolytic systems mediating maturation-associated degradation in reticulocytes between these two species. Differences in erythrocyte P2X7 activity between these two species are unlikely to be due to altered expression of splice variants. Previous immunoblotting studies using an antibody to the extracellular loop of P2X7, which is predicted to bind all known splice variants of canine P2X7 (URL: http://www.ncbi.nlm.nih.gov/gene/448778) and human P2X7[23,24], demonstrated only the full-length receptor in erythrocytes from both species[12]. Notably, the lifespans of canine and human erythrocytes are similar (approximately 115 d)[25] suggesting that P2X7-induced PS exposure in erythrocytes is unlikely to influence the removal of senescent cells.

In the current study, ATP caused visible hemolysis of canine erythrocytes, however this was not formally investigated. We have previously observed that 24 h ATP incubation induces a small but significant amount hemolysis of erythrocytes from English springer spaniels compared to those incubated in the absence of ATP (16% *vs* 1%, respectively)[12]. Future studies are required to explore if this ATP-induced hemolysis is mediated by P2X7 or other purinergic receptors, such as P2X1 or P2Y1, which can also mediate hemolysis[20,21]. Also, it remains unknown if 24 h ATP incubation causes other changes in erythrocyte morphology. Five minutes incubation with 1 mmol/L ATP of beagle erythrocytes increases cell viscosity as assessed by filterability of packed cells, but not changes in cell shape as observed by light microscopy[26]. Therefore, further studies could explore if activation of P2X7 or other purinergic receptors alters canine erythrocyte morphology.

In conclusion, the current study indicates that P2X7 activation induces PS exposure in canine erythrocytes and that this phenomenon is common to many, if not all, dog breeds. The physiological importance of P2X7-induced PS exposure in canine erythrocytes, as for human erythrocytes, remains to be established. The tendency of human erythrocytes to undergo ATP-induced PS exposure does not change with erythrocyte age[11], further supporting the concept that P2X7-induced PS exposure in erythrocytes is unlikely to be involved in the removal of aged cells. Instead, it remains plausible, that P2X7-induced PS exposure in erythrocytes is responsible for the clearance of these cells during cell stress, damage or disease. Alternatively, but not mutually exclusive to this point, P2X7-induced PS exposure in erythrocytes may facilitate thrombus formation to promote wound healing and immunity during tissue injury or infection, or to inadvertently cause vasocclusion in disorders such as malaria, sickle cell disease or diabetes. The robust PS exposure in canine erythrocytes following P2X7 activation will provide a valuable experimental model to understand further the role of this receptor in red blood cell biology. Finally, whilst PS exposure is routinely reported in canine platelets[27,28] and to some extent canine leukocytes[29,30], to the best of our knowledge PS exposure in canine erythrocytes is limited to our preliminary[12] and current observations. Thus, these studies support a rationale for exploring the physiological and pathophysiological roles and consequences of PS exposure in erythrocytes within dogs.

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COMMENTS

Background

Exposure of phosphatidylserine (PS) in erythrocytes has roles in erythrocyte clearance and thrombus formation. Activation of P2X7 by extracellular adenosine triphosphate (ATP) induces PS exposure in human erythrocytes, but whether this process occurs in erythrocytes from dogs was unknown. Therefore this study aimed to determine if ATP can induce PS exposure in erythrocytes from dogs and if so, whether this process is mediated by activation of P2X7.

Research frontiers

The mechanisms by which PS exposure on dog erythrocytes and the function of P2X7 on these cells occurs remain poorly characterised. Moreover, there are limited reports of PS exposure on dog erythrocytes in any context.

Innovations and breakthroughs

This study demonstrated that extracellular ATP causes PS exposure in dog erythrocytes from multiple breeds and that this process is mediated by activation of P2X7.

Applications

This study suggests that P2X7-mediated PS exposure on erythrocytes may have important roles in red blood cell biology in dogs. This may have potential therapeutic or biomarker applications. Moreover, the relatively high amount of P2X7-mediated PS exposure on dog erythrocytes may provide a model to study this process, including its biological significance, in greater detail.

Terminology

PS is a phospholipid that is predominately localized to the inner layer of the lipid bilayer of the plasma membrane of healthy cells, but can become localized to the outer layer (exposed) following cellular activation. Annexin V is a PS-binding protein that can be conjugated to a fluorescent label and used to study cellular PS exposure by fluorescent techniques such as flow cytometry. The P2X7 receptor is a plasma membrane ligand-gated channel activated by extracellular ATP.

Peer-review

It is a well written interesting paper studying ATP-induced PS exposure, which has potential roles in erythrocyte clearance and thrombus formation, from various dog breeds and showing that this process is mediated by P2X7 activation.

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CASE REPORT

Successful lower leg microsurgical reconstruction in homozygous sickle cell disease: Case report

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Author contributions: Posso C and Cuéllar-Ambrosi F contributed to the acquisition of data and writing of the manuscript.

Institutional review board statement: This case report was reviewed by and adhered to the standards set forth by the Institutional Review Board of the Clinic León XIII Ips University of Antioquia, Colombia.

Informed consent statement: The anonymity of the identity of the patient involved in this study was not compromised in the case report. The patient provided written informed consent for publication of these data.

Conflict-of-interest statement: Both authors declare no conflicts of interests in relation to this study or its publication.

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Abstract

We present an 18-year-old female with sickle cell disease, who presented with an extensive lower leg ulcer over a 12-year course of the disease. Definitive reconstruction was made using a free latissimus dorsi flap and split-skin grafts. One week before the surgery, the plasmapheresis protocol and blood transfusion were administered, in order to achieve a hemoglobin S level of $\leqslant$ 30%. Intraoperatively, the flap pedicle was rinsed with plasminogen activator inhibitor-1 until the thrombolytic agent was obtained from the comitant vein; after the arterial flow had been released, an intravenous bolus dose of heparin (2000 U) was administrated. No vascular complications occurred. Postoperatively, the patient received a 10-d course of hemodilution and a 14-d course of full-dose anticoagulation. After 8 mo postoperatively, the patient was able to walk and run, and showed complete wound healing. This case indicates that sickle cell disease is not a contraindication to free tissue transfer; however, the complications, their rate and overall outcomes for these cases are not yet clear. Herein, we provide an algorithm based on our clinical experience in this type of case and treatment, including several recommendations that may help to reduce thrombosis risk and systemic complications.

Key words: Sickle cell disease; Free flaps; Success rate; Microsurgical reconstruction; Ulcer

Core tip: This is a case report of a successful microsurgical

reconstruction in a patient with sickle cell disease who presented with an extensive lower leg ulcer during a 12-year course of the disease. We provide several recommendations for plasmapheresis and blood transfusions before the surgical reconstruction, and the anticoagulation protocol during the procedure and the postoperative period. This case description is intended to increase our colleagues' motivation to perform microsurgical reconstruction with a safer approach in the presence of hematologic diseases with elevated risk of thrombosis.

Posso C, Cuéllar-Ambrosi F. Successful lower leg microsurgical reconstruction in homozygous sickle cell disease: Case report. *World J Hematol* 2016; 5(4): 94-98 Available from: URL: http://www.wjgnet.com/2218-6204/full/v5/i4/94.htm DOI: http://dx.doi.org/10.5315/wjh.v5.i4.94

INTRODUCTION

The introduction of microsurgery has opened new fields for the reconstructive surgeon, so that we are now prepared to face even more challenging cases. During vascular anastomosis in microsurgical procedures, there is always risk of thrombosis, even in experienced hands and at prestigious institutions[1,2], but especially in cases of lower leg reconstruction. Patients with hematologic diseases and hypercoagulability are usually associated with high rates of complications, including anastomotic thrombosis and flap loss[3]. Clinical experience with free flaps and sickle cell disease is limited, and clear recommendations are not available.

Herein, we describe our clinical experience with a patient with sickle cell disease, who presented with a chronic ischemic lower leg ulcer that was reconstructed successfully with a free flap.

CASE REPORT

An 18-year-old Afro-American female from Chocó, Colombia, with known homozygous sickle cell disease consulted with our department regarding an extensive lower leg ulcer that had presented during a 12-year period. During that time, the patient had also presented with multiple episodes of limited osteomyelitis and soft tissue infections, for which she had been treated with systemic antibiotics; however, at no time had a real debridement and definitive reconstruction been performed because of the risk of complications (Figure 1).

One week before the microsurgical reconstruction, we started the patient on a plasmapheresis protocol and blood transfusion, with the aim of achieving a hemoglobin S level of ≤ 30%. An extensive soft tissue debridement and an anterior tibial decortication were performed subsequently, and no obvious bone defect was created. Bone and soft tissue cultures were taken; the analysis of which provided negative results. One

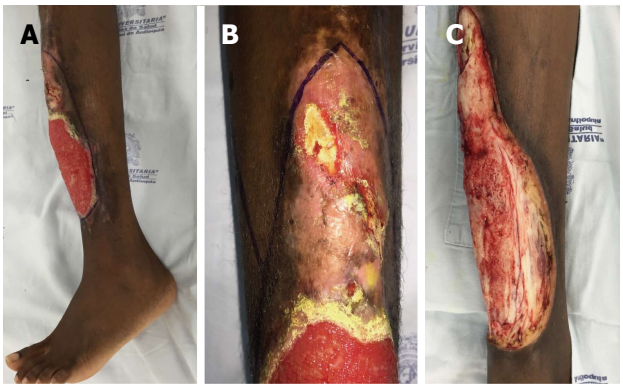

Figure 1 Ulcer on lower leg of the patient with sickle cell disease. A: Soft tissue defect; B: Detail of bone exposure; C: After extensive bone and soft tissue debridement.

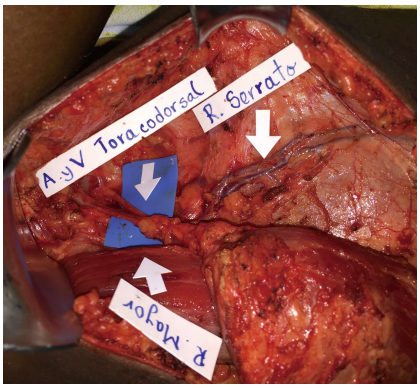

Figure 2 Vascular anatomy of the free flap.

week later, a free latissimus dorsi flap surgery was performed to cover the soft tissue defect (20 cm × 8 cm; Figure 2). The anterior tibial vessels were determined to be compromised at the middle-third of the lower leg. Before the anastomosis, the flap pedicle was rinsed using plasminogen activator inhibitor-1 (commonly known as PAI-1), until the thrombolytic agent was obtained from the comitant vein. An end-to-end anastomosis was then made between the posterior tibial artery and the thoracodorsal artery, and only one comitant vein was anastomosed. The venous anastomosis was made first following the arterial anastomosis, and later on an intravenous bolus dose of heparin (2000 U) was administered. The total ischemia time was 40 min. A partial skin graft was made to cover the muscle (Figure 3).

During the postoperative period, hemodilution was administered over a 10-d course; no vascular complications occurred. A 14-d course of full-dose anticoagulation (low-molecular-weight heparins) was administered as well. After 3 wk, the patient presented partial loss of the skin graft due to a superficial infection. The patient was admitted to the hospital for intravenous treatment with ciprofloxacin (400 mg/d). After 10 d, a second skin graft was made and complete healing was achieved. The final clinical result is shown in Figure 4,

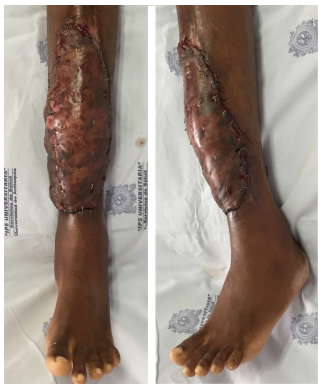

Figure 3 Immediate postoperative result. The muscle is shown from two angles covered with the split-thickness skin graft.

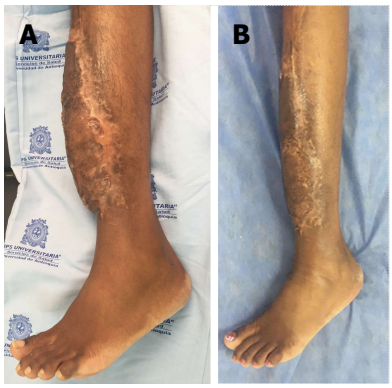

Figure 4 Follow-up postoperative results. A: After 3 mo of follow-up; B: After 8 mo of follow-up.

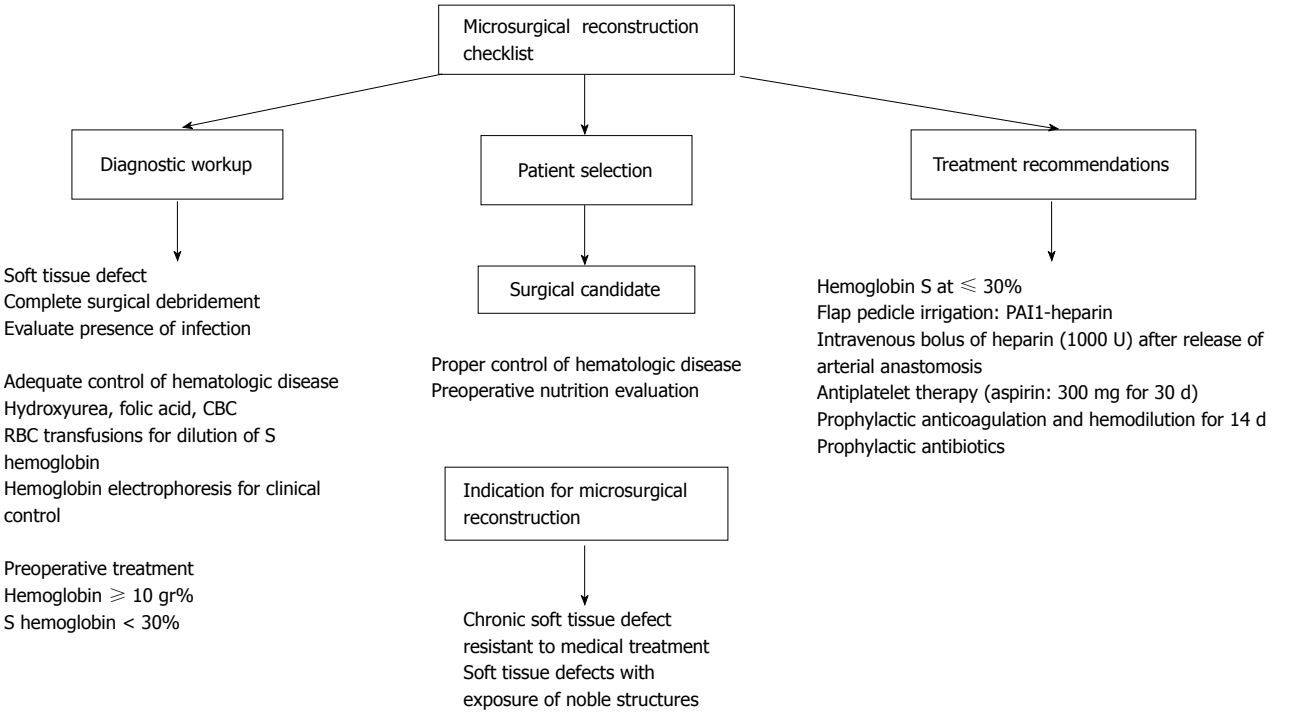

Figure 5 Algorithm treatment strategies for sickle cell disease.

after 3 mo and 8 mo of follow-up.

DISCUSSION

The sickle cell trait (SCT) results from the inheritance of one normal hemoglobin gene (HbA) and one mutated beta-globin gene (a sickle hemoglobin gene, HbS). In sickle cell disease, however, two sickle hemoglobin genes are inherited. The incidence of SCT and sickle cell disease in individuals living in the geographic region of Chocó, a Northwest Colombian area with a high density of Afro-American peoples, is 11%; intriguingly, vascular events are less frequent in these cases due to geographic adaptations of the sickle gene[4]. Increased erythrocyte sickling occurs under conditions of hypoxia, acidosis, dehydration and hypothermia[5]. SCT has been associated with an increased rate of exercise-related deaths, fetal loss, pre-eclampsia and venous thromboembolism.

Patients with SCT or sickle cell disease are usually considered a higher risk group for microsurgical reconstructions, and there is some pessimism among microsurgeons when these procedures are required[6]. In particular, rates of free flap loss in lower extremity reconstructions are higher compared to those in head and neck reconstruction, where the accepted rate of failure can be up to 5%. Failure rates reported in lower leg reconstruction, in contrast, varies from 6% to 15%, depending on the case series[7].

There is growing evidence from reports of clinical experiences involving patients with hematologic diseases and microsurgery[8], but most of the case series have thus far included patients with very different types of disorders (*i.e.,* various etiologies and risks

of thrombosis or bleeding), such as an increased number of blood elements responsible for hemostasis, abnormal blood elements or abnormality within the coagulation cascade. If we consider patients with sickle cell disease exclusively, only a few cases have been reported to date, some of which include flap loss[9,10]; hence, the question remains: Can we compare different hematologic diseases and outcomes in microsurgery?

According to our experience, there are several recommendations that should be included in this particular group of patients undergoing microsurgical reconstruction (Figure 5). In the first place, control of the medical condition is essential and the hematologist should work closely with the reconstructive surgeon. Normally, the patient receives multiple transfusions to maintain the HbS level well below the recommended 30% level for a free flap surgery so that the risk of anastomosis occlusion can be minimized[11]. On the other hand, it has been strongly recommended to use prophylactic anticoagulation therapy, but there is no evidence that this treatment will avoid any type of vascular complication. The ideal duration and type of therapy are not yet known.

Regarding the microsurgical technique, Ozkan *et al*[12] reported some strategies that would help to avoid complications, but we disagree on some of them. First, proper flap selection does not depend on whether or not your patient has any hematologic disease; reliable anatomy is always desirable, but donor site morbidity, extension and location of the soft tissue defect should be always considered. Ozkan *et al*[12] also recommended large recipient vessels, but what it is really important is to perform the vascular anastomosis in a healthy zone, avoiding inflammation of the vessel wall.

In conclusion, microsurgery transfer provides a well-vascularized tissue to solve extensive soft-tissue defects in a single procedure. In patients with SCT or sickle cell disease, complications such as thrombosis, bleeding, infection or poor wound healing may occur. This particular group of patients should be evaluated carefully before surgery so as to modify any potential risk factor. We have now demonstrated that it is possible to perform a successful free flap in one of these patients, but more extensive and closer monitoring in the postoperative period and a longer regimen of anticoagulation agents might be indicated. Moreover, this is a single-case report and larger case series are needed to clearly establish outcomes and rate of complications.

COMMENTS

Case characteristics

An 18-year-old female with medical history of sickle cell disease presented with a 12-year history of skin ulcer in her left leg and multiple episodes of limited osteomyelitis and soft tissue infections. The ulcer was reconstructed using a free flap.

Clinical diagnosis

A soft tissue defect was located over the anterior surface of the lower leg with bone exposure (20 cm × 8 cm).

Differential diagnosis

Chronic osteomyelitis, vascular ulcer.

Laboratory diagnosis

The hemoglobin level at admission was consistent with severe anemia.

Imaging diagnosis

A vascular occlusion was present in the anterior tibial artery.

Pathological diagnosis

After surgical debridement, bone culture was negative.

Treatment

Definitive soft tissue reconstruction was made using a free muscular flap and split-thickness skin graft.

Related reports

Microsurgical reconstruction in patients with hematologic diseases has not been reported frequently in the medical literature. Furthermore, if the authors consider patients with sickle cell disease exclusively, very few case reports are available, some of which include flap loss. There are no clear recommendations on how to decrease the risk of pedicle thrombosis in this patient population.

Term explanation

Sickle cell disease is an inherited red blood cell disorder, in which two sickle hemoglobin genes (HbS) are inherited. Increased erythrocyte sickling and secondary thrombosis occurs under special conditions, such as hypoxia, acidosis, dehydration and hypothermia, causing vascular compromise and skin necrosis in some cases. Patients with the sickle cell trait or disease are usually considered a higher risk group for microsurgical reconstructions, and preoperative control of the disease and a different surgical protocol should be included on order to avoid vascular complications.

Peer-review

This is an interesting and well written clinical case report.

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